

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Henry Nicolas Jabbour *et al*
Serial No: 10/511,480
Filed: April 10, 2003
Title: FP Receptor Antagonists or PGF2 alpha Antagonists for Treating Pathological Conditions of the Uterus
Group Art Unit: 4173
Examiner: Marcos Sznaidman

SECOND DECLARATION OF HENRY NICOLAS JABBOUR UNDER 37 C.F.R. § 1.132

I, Henry Nicolas Jabbour, pursuant to 37 C.F.R. § 1.132, declare:

1. I am one of the inventors of the above-mentioned patent application. I am a Programme Leader and Senior Scientist at the UK Medical Research Council Human Reproductive Science Unit in Edinburgh, Scotland. I have worked in the field of reproductive biology and health for approximately 20 years. My resumé was attached to my first declaration dated February 24, 2009.
2. I have read the Office Action dated April 28, 2009 and I understand that the examiner is continuing to reject Claims 1, 3-5, 9 and 12-13 of the application as failing to comply with the enablement requirement. I am presenting this declaration to demonstrate further the credibility of the use of FP receptor antagonists in treating pathological conditions of the uterus, and in particular uterine carcinoma, endometriosis and fibroids. In particular, I am further emphasising the importance of the data presented with my first declaration which confirms that FP receptor antagonists are useful in treating pathological conditions of the uterus, as described in the patent application, and I am providing further supporting data in Annexes 1 and 2 attached hereto.
3. A copy of Sales *et al* (2005) *Cancer Res.* **65**, 7707-7716, which was published after the patent application was filed, was attached as Annex 3 to my first Declaration. I am the corresponding author of that paper and the work leading to the paper was done under my supervision in my laboratory, and builds on the work described in the patent application (which shows elevated expression of FP receptor in uterine carcinoma).
4. The work presented in Sales *et al* shows that elevated FP receptor and VEGF expression co-localized in glandular and epithelial cells lining the blood vessels in endometrial (uterine) adenocarcinomas. Furthermore, it shows that PGF2 α (the natural ligand of the FP receptor) can cause rapid transphosphorylation and activation of the EGF receptor, and activation of MEK signalling via the FP receptor resulting in an increase in VEGF promoter activity, expression of VEGF mRNA and secretion of VEGF protein, all of which are consistent with a role for the FP receptor in stimulating blood vessel formation (angiogenesis) in endometrial (uterine) cancer (see Abstract).
5. Sales *et al* investigates the effect of a specific FP receptor antagonist, namely AL8810, in a way that is directly relevant to establishing that FP receptor antagonists have relevant activities for the treatment of uterine cancer, endometriosis and

fibroids. In particular, it investigates the effect of the FP receptor antagonist on the PGF2 α signalling to VEGF in endometrial adenocarcinoma using endometrial adenocarcinoma (Ishikawa) cells and using endometrial adenocarcinoma tissue explants.

6. Sales *et al* discloses that the effects of PGF2 α on the FP receptor could be abolished by treatment of cells with a specific FP receptor antagonist, AL8810. This can be seen from page 7711, column 2, and from Figure 4b, lane 3, which notes that the activation of ERK1/2 was abolished by cotreatment with AL8810 (ie treatment with PGF2 α and AL8810). Figures 5A and 5B further show AL8810 treatment prevents PGF2 α -FP mediated increase in VEGF mRNA expression and protein secretion in Ishikawa cells (see also page 7712, column 2 to page 7713, column1).
7. Furthermore, similar effects were found when endometrial adenocarcinoma explants were treated with AL8810 (see, in particular, page 7713, column 2; and also see Figure 6a, lane 3).These data show that AL8810 abolishes PGF2 α mediated increase in tyrosine phosphorylation of EGFR. EGFR is known to be involved in many cancers, and these results show that FP receptor induces the activity of EGFR in endometrial cancer, and more importantly the results show that an antagonist of the FP receptor inhibits the activity of EGFR induced by PGF2 α . Hence, the FP receptor antagonist inhibits not only the FP receptor, but also indirectly the activity of another receptor (EGFR) with renowned roles in cancer. Moreover, Figure 6b demonstrates that AL8810 treatment prevents PGF2 α -FP mediated increase in VEGF mRNA expression in endometrial adenocarcinoma explants (see also page 7713, column 2 to page 7714, column 1).
8. My laboratory has carried out further studies on the role of FP receptors in endometrial (uterine) carcinoma which are described in Annexes 1 and 2 of this Declaration.
9. Annex 1 is a manuscript by Keightley *et al* entitled "F-Prostaglandin receptor regulates endothelial cell function via Fibroblast Growth Factor-2" which has been submitted for publication in the journal *Biomed Central Cell Biology*. This manuscript describes work carried out under my supervision and I am the corresponding author. The work in this manuscript shows that F-prostaglandin receptor (the FP receptor) regulates endothelial cell function via Fibroblast Growth Factor-2 (FGF-2). Angiogenesis (the formation of new blood vessels) has an important part to play in endometrial (uterine) carcinogenesis and this requires the processes of endothelial cell differentiation (or network formation) and proliferation, both of which are therefore targets for therapeutic intervention.
10. Particularly relevant is the first full paragraph on page 16 of Annex 1 which describes the effect of PGF2 α -FP receptor interaction in endothelial network formation. It can be seen that the FP receptor antagonist AL8810 prevented an increase in endothelial network formation, which is necessary for angiogenesis, again clearly supporting the use of FP receptor antagonists for treating endometrial (uterine) carcinoma, which requires angiogenesis.
11. Annex 2 is Wallace *et al* (2009) *Cancer Res.* **69**, 5726-5733 which describes work carried out under my supervision, and I am the corresponding author. It shows that FP receptor upregulates expression of CXCL1, a chemokine involved in the inflammatory pathways in tumours, and this is inhibited by treatment with the FP receptor antagonist AL8810 (see, in particular, the paragraph spanning pages 5728 and 5729). The paper goes on to show that the CXCL1 upregulated by FP receptor results in increased attraction of neutrophils into the uterine cancer microenvironment which is believed to enhance angiogenesis and promote metastasis (see third full

paragraph on page 5729). Hence, antagonism of the FP receptor which blocks the expression of CXCL1, will reduce inflammatory pathways in uterine cancer and suppress the influx of neutrophils.

12. Consistent with the description in the present application (see in particular the conclusion to Example 1 on page 34 lines 8 to 24), the results in Sales *et al* and in Annexes 1 and 2 hereto confirm that an FP receptor antagonist can play a direct role in treating a pathological condition of the uterus, such as uterine carcinoma. AL8810 is one of the FP receptor antagonists specifically exemplified in the patent application at page 9, lines 1-7.
13. Annex 4 of my first Declaration provides additional data which show that FP receptor expression is consistently higher in the endometrium of women with fibroids, during all phases of the menstrual cycle, than those without fibroids.
14. In this study, which was conducted in my laboratory under my supervision, endometrium was collected from women with fibroids and those without, RNA was extracted from these tissues and then we assessed the level of expression of the FP receptor from the two groups of women by a technique known as reverse transcriptase polymerase chain reaction (this technique allows one to make direct comparisons of the levels of expression of the receptor in different women). In the endometrium of women with fibroids, the level of expression of the FP receptor was consistently higher.
15. Taking into account our knowledge of the mechanism of action of the FP receptor and its rôle in exacerbating vascular function, antagonizing the action and signalling of this elevated FP receptor in the endometrium of women with fibroids is expected to be an effective therapeutic intervention strategy that may limit the blood loss that is associated with this pathology. For the reasons discussed above, this gives further credibility that FP receptor antagonists are useful in treating pathological conditions of the uterus, including fibroids.
16. I maintain my conclusion that the data presented in the present application and obtained subsequent to the filing date confirm that several uterine pathological conditions, including endometriosis, uterine carcinoma, and fibroids, all involve enhanced FP receptor expression; and, consistent with the description in the application for treating such uterine pathological conditions, treatment of uterine carcinoma explants with an FP receptor antagonist was shown to be effective in preventing FP receptor mediated expression of pro-angiogenic factors like VEGF and in inhibiting endothelial network formation, which are essential features of angiogenesis in uterine carcinoma.
17. The results therefore confirm the efficacy of treating pathological conditions of the uterus using FP receptor antagonists. Given the demonstrated efficacy of treating uterine carcinoma explants with an FP receptor antagonist, persons of skill in the art would fully appreciate that a female individual having a pathological condition of the uterus, such as uterine carcinoma, endometriosis, uterine fibroids, or any other pathological conditions of the uterus that are associated with abnormal growth of the myometrium or endometrium, can be effectively treated for the condition by administering to the affected individual an FP receptor antagonist.

18. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed at Edinburgh, Scotland

This 14th day of August 2009

Henry Jabbour
Henry Nicolas Jabbour

IN THE MATTER of US Patent Application
Serial No 10/511,480

Annex 1

Keightley *et al* manuscript entitled "F-Prostaglandin receptor regulates endothelial cell function via Fibroblast Growth Factor-2"

Signed

KENNY MCBRIDE

Dated

14th August 2001

F-Prostaglandin receptor regulates endothelial cell function via Fibroblast Growth Factor-2.

Margaret C. Keightley, Pamela Brown, Henry N. Jabbour[§] and Kurt J. Sales

MRC Human Reproductive Sciences Unit, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh, EH16 4TJ.

ABBREVIATED TITLE: FP receptor signaling regulates endothelial cell function

KEY WORDS: Prostaglandin, FP receptor, FGF2, COX-2, angiogenesis, cancer

§Address all correspondence to: Dr Henry N. Jabbour, MRC Human Reproductive Sciences Unit, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh, EH16 4TJ, UK. Telephone: 131-2426220, Fax: 44-131-2426231. E-mail address: H.Jabbour@hrsu.mrc.ac.uk

Abstract

Prostaglandin (PG) $F_{2\alpha}$ is a key regulator of endometrial function and exerts its biological action after coupling with its heptahelical G protein-coupled receptor (FP receptor). We have shown that PGF $_{2\alpha}$ -FP receptor signaling in endometrial adenocarcinoma cells can up-regulate angiogenic factors including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF2). In the present study, we investigated the paracrine effect of FGF2, produced via PGF $_{2\alpha}$ -FP receptor signaling in endometrial adenocarcinoma cells stably expressing the FP receptor (Ishikawa FPS cells), on endothelial cell function. Conditioned medium (CM) was collected from FPS cells after 24hrs treatment with either vehicle (V CM) or 100nM PGF $_{2\alpha}$ (P CM). Treatment of human umbilical vein endothelial cells (HUVECs) with P CM significantly enhanced endothelial cell differentiation (network formation) and proliferation. Using chemical inhibitors of intracellular signaling, we found that endothelial network formation was mediated by FGF2-FGFR1, following phosphorylation of ERK1/2 and subsequent induction of COX-2, release of PGF $_{2\alpha}$ and activation of endothelial FP receptors. Whereas P CM stimulation of endothelial cell proliferation occurred via an FGF2-FGFR1-ERK1/2 dependent mechanism involving activation of the mTOR pathway. Taken together, we have elucidated two molecular pathways whereby FGF2, released from epithelial cells following PGF $_{2\alpha}$ -FP receptor interaction, regulates endothelial cell network formation and proliferation.

Introduction

Endometrial adenocarcinoma, originating from the glandular epithelial cells of the uterine endometrial lining, is one of the most prevalent cancers amongst women in the Western world [1, 2]. Although it is a disease which particularly occurs in post menopausal women, where progression of endometrial adenocarcinoma is considered to be under hormonal control by oestrogen and progesterone [3, 4], recent evidence suggests that mutations in oncogene expression may play a role in the etiology of the disease [5]. Data generated in our laboratory and others have ascertained a role for the cyclooxygenase (COX)-prostaglandin (PG) axis in the progression of endometrial adenocarcinomas by increasing cell proliferation and the secretion of angiogenic growth factors [6, 7]. This is in accordance with other neoplastic disorders where over-expression of COX enzymes and biosynthesis of prostaglandins has been shown to control benign and neoplastic diseases by promoting cellular proliferation [8], inhibiting apoptosis [9] and enhancing angiogenesis [10] via the activation of prostanoid receptors. However, the molecular mechanisms mediating the role of prostaglandins in regulating vascular function and angiogenesis are still poorly defined.

Angiogenesis is the process of endothelial cell sprouting from an existing vasculature towards cancer cells [11] and is required by any tumour larger than 2mm in diameter [12]. The proposed mechanism of angiogenesis suggests that tumour cells secrete stimulatory factors which act in a paracrine manner on surrounding blood vessels, immune cells and fibroblasts to promote the proliferation, differentiation and migration of endothelial cells towards the stimulus [12, 13]. These tumour stimulatory factors include vascular endothelial growth factor (VEGF-A) and fibroblast growth factor 2 (FGF2). In

human endometrial adenocarcinomas VEGF-A and FGF2 expression and secretion are elevated [14-16] and both VEGF-A and FGF2 can stimulate angiogenesis in xenografts *in vivo* [17, 18].

In a previous study we demonstrated elevated expression of the FP receptor, FGF2 and the FGF2 receptor 1 (FGFR1) in neoplastic endometrial epithelial and vascular cells and ascertained a role for the FGF2, produced by PGF_{2 α} -FP receptor signaling, on epithelial cell proliferation [14]. In this study we have shown that conditioned medium from PGF_{2 α} treated Ishikawa cells stably expressing the FP receptor to the levels observed in endometrial adenocarcinoma (Ishikawa FPS cells), can increase endothelial cell differentiation (network formation) and proliferation. We investigated the mechanism by which conditioned medium mediated the PGF_{2 α} -FP receptor effects on endothelial network formation and proliferation. We demonstrate that FGF2-FGFR1 signaling in endothelial cells induces the phosphorylation of extracellular signal-regulated kinase (ERK1/2), activation of COX-2 and promotes the secretion of PGF_{2 α} . Following its release from endothelial cells, PGF_{2 α} promotes endothelial network formation in an autocrine/paracrine manner, via the endothelial FP receptor. By contrast FGF2-FGFR1 signaling to ERK1/2 promotes endothelial cell proliferation by a separate mechanism via the mammalian target of rapamycin (mTOR) pathway. Taken together, our data highlight two molecular mechanisms by which PGF_{2 α} can regulate endothelial cell function and propose a mechanism whereby PGF_{2 α} -FP receptor signaling can regulate endothelial cell function in endometrial adenocarcinomas.

Materials and Methods

Reagents

The FGF2 antibody recognising the 18kDa isoform of FGF2 (sc1360) and the FGFR1 antibody (sc-121) were purchased from Santa Cruz Biotechnology (Autogen-Bioclear, Wiltshire, UK). Arachidonic acid (AA), PGE₂, PGF_{2α}, AL8810 and indomethacin were purchased from Sigma Chemical Co. (Dorset, UK). PD98059, SU4984, LY294002, PP2, wortmannin and rapamycin were purchased from Calbiochem (Nottingham, UK). Recombinant FGF2 peptide was purchased from PeproTechEC Ltd. (London, UK). Anti-phospho-p42/44 ERK (9101) and anti-p42/p44 ERK were purchased from Cell Signaling Technologies/New England Biolabs (Hertfordshire, UK).

Cell culture.

Ishikawa cells stably expressing FP receptor (Ishikawa FPS cells) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Paisley, UK) with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin solution as described previously [15]. Human umbilical vein endothelial cells (HUVECs) (Lonza, Walkersville, USA) were cultured in Endothelial Basal Medium (EBM-2) with 2% FBS and growth supplements (VEGF, FGF, PGDF, IGF, EGF, ascorbic acid, heparin and gentamycin) subsequently referred to as Endothelial Growth Medium (EGM-2) (Lonza, Walkersville, USA). Under experimental conditions HUVECs were incubated with EBM-2 plus 1% FBS with the addition of ascorbic acid and gentamycin (EBM1%) (Lonza, USA).

Conditioned medium

Conditioned medium (CM) was prepared as described previously [14]. Briefly, FPS cells were seeded at a density of 2×10^6 cells and allowed to adhere before serum-starvation for 24hrs. Thereafter, cells were treated with 20mls of DMEM containing 8.4 μ M indomethacin in the presence of 100nM PGF_{2 α} or vehicle for 24hrs to create PGF conditioned medium (P) or vehicle conditioned medium (V). Conditioned medium from three independent experiments was pooled, aliquoted and stored at -20 °C until required. FGF2 was immunoneutralised from the PGF_{2 α} conditioned medium by overnight incubation with 0.5 μ g/ml FGF2 antibody. The immune complex was removed by 4hr incubation with 20 μ l of a 50% protein A plus G slurry (Calbiochem). Conditioned medium immunoneutralised with Goat IgG was used as a control. Immunoneutralised CM was aliquoted and stored at -20°C until use. The FGF2 content in the CM before and after immunoneutralisation was confirmed by ELISA as described previously [14].

Network assays

Network assays were carried out using 12-well Transwell plates (Corning Costar, Cambridge, UK). The upper chambers were coated with 80 μ l of growth factor (GF)-reduced Matrigel (BD Biosciences, MA, USA) in the absence/presence of SU4984, PD98059, rapamycin, wortmannin, LY294002 or AL8810 and incubated at 37°C for 30 mins to allow thin gel formation. HUVECs were plated onto the gel (2.5×10^4 cells/well) in EBM 1%. In the lower chamber V CM or P CM was added. Transwell plates were incubated at 37°C in a 5% CO₂ atmosphere for 16hrs. Subsequently, cell networks were fixed with 100% ice cold methanol and stained with haematoxylin. To assess network

formation, each well was divided into 5 sections. Hotspots of each section were photographed, 5 photos per well at x10 magnification, using an inverted microscope and camera (Axiovert 200, Carl Zeiss, Germany). The number of network branches was counted blind. Experiments were repeated at least four times in duplicate. Fold difference was determined by dividing the value obtained from P CM treated cells by the value obtained from V CM treated cells. Data are represented as percentage increase in network formation with V CM =100% and are presented as mean \pm SEM.

Proliferation assay

HUVECs were seeded in 96-well plates at 3000 cells/well. Following attachment, cell medium was replaced with EBM1% for 3 hours. Cells were then treated with CM, diluted 1:1 (v/v) with EBM1%, in the absence/presence of SU4984 (20 μ M), PD98059 (50 μ M), rapamycin (100ng/ml), wortmannin (200nM), LY294002 (50 μ M), AL8810 (50 μ M) or FGF2-immunoneutralised CM. Treatments were replaced three times during the 96hr incubation. Proliferation was determined using the CellTitre96AQueous One Solution Proliferation Reagent (Promega, Southampton, UK) as per the manufacturer's instructions. The experiments were repeated three times in quadruplicate. Fold difference was determined by dividing the absorbance obtained by P CM treated cells by the absorbance obtained by V CM treated cells. Data are represented as percentage increase in proliferation with V CM = 100% and are presented as mean \pm SEM.

Western Blot analysis

HUVECs were seeded at 2×10^5 cells per 60mm diameter dish and left to adhere for 24hrs before serum starvation overnight. GF-starved HUVECs were treated with P CM or V CM for the time indicated in the figure legend, rinsed with ice-cold phosphate-buffered saline and lysed for 20mins with protein lysis buffer containing inhibitor cocktail mix as described previously [19]. Protein concentration was determined with a protein assay (BioRad, Hercules, CA) and approximately 16 μ g of protein was resolved and immunoblotted as previously described [20]. Immunoblots were blocked in Odyssey Blocking bufferTM (LI-COR Biosciences, Cambridge, UK) before overnight incubation with primary phospho-p42/44 and p42/44 antibodies (diluted 1:1000 in Odyssey blocking buffer) at 4°C. The following day, blots were washed and incubated with the goat anti-mouse IRDyeTM 800 (1:10,000) (Rockland Immunochemicals Inc., Gilbertsville, PA, USA) and goat anti-rabbit Alexafluor 680 (1:5000) (Invitrogen) for 60 minutes at room temperature.

Immunoreactive proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences). ERK1/2 phosphorylation was calculated by dividing the value obtained from the phosphorylated ERK1/2 channel (700nm) by the value obtained from total ERK1/2 channel (800nm) and expressed as fold above vehicle controls. Results are expressed as mean \pm SEM from at least four independent experiments.

Taqman quantitative RT-PCR

Taqman RT-PCR was performed as described previously using sequence specific primers and probes designed to span an intron [19, 21, 22]. Briefly, HUVECs were seeded at 5×10^4 cells per well with EBM1% in a 6 well plate. Following overnight serum starvation, cells were treated with V CM or P CM, diluted 1:1 (v/v) with EBM 1% in the absence or presence of SU4984 (20 μ M) or PD98059 (50 μ M) for the time indicated in the figure legends. RNA was extracted, reverse transcribed and RT-PCR performed using the ABI Prism 7900 as described previously [14]. FP, EP2, COX-1 and COX-2 mRNA were normalized using ribosomal 18S as an internal control. Experiments are representative of at least five independent experiments. Fold difference was determined by dividing the value obtained from P CM treated cells by the value obtained from V CM treated cells and represented as mean \pm SEM.

PGF_{2 α} /PGE₂ ELISA

HUVECs were seeded at a density of 5×10^4 cells per 35mm diameter dish and serum starved overnight. Thereafter cells were treated with V CM or P CM diluted 1:1 (v/v) with EBM 1% plus 6ug/ml arachidonic acid for the time indicated in the figure legend. After treatment CM was collected and stored at -20° C until required. PGF_{2 α} and PGE₂ secretions in the culture media were assayed by ELISA as described previously [23, 24]. Experiments were repeated three times. The data are presented as mean \pm SEM.

Short hairpin DNA constructs and preparation of adenoviral stocks

To generate FP receptor knockdown vectors, oligonucleotides encoding short hairpin transcripts were annealed and individually cloned into the adenovirus shuttle vector pDC316. The start codon of the FP receptor (NM_000959) was used as a reference and labeled as basepair 1. Thus the target sequences corresponded to 306 and 478bp downstream and included a scrambled (Scr) negative control: Sh306: 5'-GCTGCGCTTCTTCAAACA; Sh478: 5'-GTGGCCTGGTAATCACTGA; Scr: 5'-TTACTCGACGCATGTGCTT. High titre stocks (<10e10 viral plaque forming units/ml, pfu/ml) were prepared using the AdMax system (Microbix Biosystems Inc., Toronto, Ontario, Canada). Briefly, an adenovirus genomic plasmid (pBHGLoxdeltaE1,3Cre) was cotransfected with the shuttle vector into HEK293 cells. After 10 days, plaques were purified and seeded into a T75 flask of HEK293 to generate the first seed. When HEK293 cells showed a cytopathic response; virus was released from the cells by three cycles of freeze thawing. This starter culture was used to produce large bulk preparations of adenovirus. Viral particles were purified using a Vivascience AdenoPack column (Generon House, Eton Wick, UK), buffer exchanged into 8 volumes of 2.5% glycerol, 20 mM Tris-HCl (pH 8) and then concentrated. The viral titre was determined using a modified Adeno-X rapid titre kit (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) with 1:1000 rabbit anti-adenovirus Serotype 5 hexon antisera (Labfrontier, Seoul, Korea).

To ablate FP receptor expression, HUVECs were seeded at a density of 3×10^5 cells/25cm² in EGM-2 and incubated with 100 viruses (either scr, sh306 or sh478) per cell (MOI) for 24hrs at 37°C in a 5% CO₂ atmosphere. The next day, the viral

infected cells were washed, trypsinised, counted and used in the network formation and proliferation assays.

Statistics

Statistical significance was assessed with one-way ANOVA and Dunnett's post hoc test using Prism 5.0 (Graph Pad, San Diego, CA). A *p*-value of less than 0.05 ($P<0.05$) was considered to be statistically significant.

Results

Endothelial cell network formation and cell proliferation are mediated via FGF2-FGFR1 signaling.

We previously demonstrated elevated expression of the FP receptor, FGF2 and FGFR1 in endometrial adenocarcinoma [14]. In addition, using a neoplastic epithelial cell line stably expressing the FP receptor to the levels observed in endometrial adenocarcinoma (Ishikawa FPS cells), we ascertained a role for FGF2, produced by PGF_{2 α} -FP receptor signaling, on epithelial cell proliferation [14]. Moreover we found that FP receptor, FGF2 and FGFR1 co-localised within the vascular endothelial cells in endometrial adenocarcinomas suggesting that PGF_{2 α} may directly and indirectly regulate endothelial cell function [14]. To determine a role for FGF2, produced by PGF_{2 α} -FP receptor interaction in endometrial adenocarcinoma cells, on endothelial cell function, we used conditioned medium (CM) from Ishikawa FPS cells treated with vehicle or 100nM PGF_{2 α} for 24 hours. The presence of FGF2 in CM from vehicle (V) and PGF_{2 α} -treated (P) Ishikawa FPS cells was confirmed by ELISA (Fig. 1A).

To assess the effects of the CM on endothelial cell function, cellular differentiation (network formation) and proliferation assays were performed using HUVECs as a model system. Treatment of HUVECs with P CM significantly increased endothelial cell network formation and proliferation compared to V CM-treated cells (Fig. 1B and 1C respectively, P<0.05). Treatment of HUVECs with P CM in the presence of the FGF2 receptor 1 (FGFR1) tyrosine kinase inhibitor (SU4984) or CM

which had FGF2 immunoneutralised (FGF2-Ab; Fig. 1A), significantly reduced endothelial network formation (Fig. 1B; $P<0.05$) and cellular proliferation (Fig. 1C; $P<0.05$), confirming that these alterations in endothelial cell function were mediated by FGF2 in the CM via FGFR1.

Next we investigated the signal transduction pathways mediating the role of FGF2 in the CM on network formation and proliferation. HUVECs were treated with P CM in the presence of cell signaling inhibitors of extracellular signal-regulated kinase (ERK1/2; PD98059), mammalian target of rapamycin (mTOR; rapamycin) or phosphoinositide-3-kinase (PI3K; Wortmannin or LY294002). We found that the P CM-induced network formation was significantly inhibited by PD98059 but not rapamycin, wortmannin or LY294002 (Fig. 2A, $P<0.05$). However, endothelial cell proliferation was inhibited by PD98059 and rapamycin but not wortmannin or LY294002 (Fig. 2B, $P<0.05$).

We confirmed that endothelial cell proliferation but not network formation was mediated by FGF2-mTOR signaling using recombinant FGF2 protein. Treatment of HUVECs with recombinant FGF2 significantly increased network formation (Fig. 2C; $P<0.05$) and proliferation (Fig. 2D; $P<0.05$). Co-treatment of cells with recombinant FGF2 protein and rapamycin had no effect on network formation, compared to recombinant FGF2 peptide alone (Fig. 2C; $P<0.05$). In contrast, rapamycin treatment significantly inhibited endothelial cell proliferation induced by the recombinant FGF2 protein (Fig. 2D; $P<0.05$) confirming that endothelial cell proliferation was mediated by P CM via the FGF-FGFR1-mediated induction of the mTOR pathway.

Conditioned medium from Ishikawa FPS cell treated with PGF_{2α} induces endothelial COX-2 and FP receptor.

FGF2 has been shown to mediate angiogenesis via COX-2 in an *in vivo* model of rat sponge implants [25] hence we investigated the effect of P CM on the expression of COX-1, COX-2, EP1, EP2, EP3, EP4 and FP receptor in endothelial cells. HUVECs were treated with V CM or P CM for 1, 2, 3, 4, 6, 16 and 24 hrs. We did not observe an alteration in expression of COX-1 (Fig. 3A), EP1, EP3 or EP4 receptor (data not shown) at any of the time points investigated in response to P CM stimulation. However, we observed a significant increase in COX-2 (Fig. 3B; P<0.05), FP receptor (Fig. 3C; P<0.05) and EP2 receptor (Fig. 3D; P<0.05) expression at 3 hours in HUVECs following treatment with P CM.

Endothelial COX-2, FP receptor and EP2 receptor expression is regulated via the FGF2-FGFR1-ERK1/2 pathway.

To determine if FGF2-FGFR1 signaling is involved in the regulation of COX-2, FP receptor and EP2 receptor expression, we co-incubated HUVECs with V CM or P CM in the absence/presence of inhibitors of FGFR1 tyrosine kinase activity (SU4984) or ERK1/2 (PD98059). Treatment of HUVECs with P CM for 3 hrs in the presence of SU4984 or PD98059 significantly inhibited the P CM-induced expression of COX-2 (Fig. 4A; P<0.05), FP receptor (Fig. 4B; P<0.05) and EP2 receptor (Fig. 4C; P<0.05), indicating that COX-2, FP receptor and EP2 receptor expression was regulated by FGF2-FGFR1 interaction via the ERK1/2 pathway.

ERK1/2 phosphorylation is regulated by FGF2- FGFR1 signaling.

We investigated P CM-mediated ERK1/2 phosphorylation in endothelial cells. HUVECs were treated with V CM or P CM for 0, 5, 10, 15, 20 and 30 mins (Fig. 5A). Treatment of HUVECs with P CM significantly increased ERK1/2 phosphorylation in a time-dependent manner which was maximal after 10 mins of stimulation, compared to V CM (Fig. 5A; P<0.05). Co-incubation of HUVECs with P CM in the presence of FGFR1 tyrosine kinase inhibitor (SU4984), c-Src inhibitor (PP2) or ERK1/2 inhibitor (PD98059) significantly reduced the P CM-stimulated phosphorylation of ERK1/2 (Fig 5B, P<0.05). However treatment of HUVECs with P CM in the presence of the PI3K inhibitor LY294002 did not significantly reduce the P CM phosphorylation of ERK1/2 (Fig. 5B). Similarly, co-incubation of HUVECs with P CM and the mTOR inhibitor, rapamycin, or the PI3K inhibitor wortmannin had no effect on ERK1/2 phosphorylation (data not shown). Treatment of HUVECs with recombinant FGF2 protein phosphorylated ERK1/2 to the levels observed with P CM (Fig. 5B).

The role of endothelial PG in the regulation of endothelial cell network formation

Since P CM treatment of HUVECs enhanced COX-2, FP and EP2 receptor expression, we examined the role of endothelial PGE₂ and PGF_{2 α} in P CM-induced endothelial cell network formation and proliferation. HUVECs were treated with V CM or P CM for 0, 3, 6, and 16 hrs and the secretion of PGE₂ (Fig. 6A) and PGF_{2 α} (Fig. 6B) was measured by ELISA. There was no significant difference in the levels of endothelial PGE₂ secreted by HUVECs in response to CM at any time point tested (Fig. 6A). In

contrast, the amount of endothelial PGF_{2 α} was elevated in HUVECs at 6 hours following P CM treatment compared to V CM treatment (Fig. 6B).

To investigate the autocrine/paracrine effect of PGF_{2 α} -FP receptor interaction in endothelial network formation, we treated HUVECs with P CM or 1 μ M exogenous PGF_{2 α} in the presence/absence of the specific FP receptor antagonist AL8810. We found that P CM and PGF_{2 α} induced a significant increase in endothelial network formation (Fig. 7A, P<0.05) but not proliferation (Fig. 7B). Co-treatment of HUVEC with P CM or exogenous PGF_{2 α} and the FP receptor antagonist AL8810 prevented an increase in network formation (Fig. 7A, P<0.05). This suggests that the PGF_{2 α} signaling through the FP receptor is involved in endothelial network formation but not proliferation.

To confirm the role of the FP receptor in P CM-induced network formation we used two separate short hairpin RNA adenoviral constructs targeted against the FP receptor (sh306 and sh478) to knockdown FP receptor expression in HUVECs. HUVECs were infected with scrambled adenovirus (scr), sh306 or sh478 for 24hrs. Efficiency of the FP shRNA in ablating receptor expression was confirmed by quantitative RT-PCR analysis (Fig. 7C). Infection of HUVECs with sh306 and sh478, significantly reduced P CM-induced network formation compared to scrambled control virus (Fig. 7D, P<0.05). In contrast, infection of HUVECs with either FP receptor shRNA adenovirus did not alter P CM-induced endothelial proliferation compared to HUVECs infected with the control scrambled adenovirus (Fig. 7E), similar to our observation with the FP receptor antagonist AL8810.

Discussion

FGF2 is one of 23 fibroblast growth factor family members and signals via one of four receptors, FGFR1, 2, 3 and 4, of which FGFR1 is most commonly expressed on endothelial cells [26]. A principal role of secreted FGF2 is to stimulate blood vessel growth although we have shown previously that it can also act as a potent autocrine growth factor to enhance epithelial cell proliferation [14]. In vitro and animal xenograft studies have shown that secretion of epithelial FGF2 in endometrial adenocarcinoma xenografts can enhance tumour growth by enhancing blood vessel size and width [27]. Furthermore antisense targeting of FGF2 in such model systems is known to reduce tissue microvascular density as well as xenograft size [28]. However the molecular mechanisms whereby FGF2 regulates vascular function are less clear.

Endothelial cell differentiation and proliferation are two of the processes required for angiogenesis [13, 29-31]. In the present study we have shown that conditioned medium from endometrial adenocarcinoma cells stably expressing the FP receptor to the levels observed in endometrial adenocarcinomas (FPS cells) and which we had shown to produce FGF2, promotes endothelial network formation (differentiation) and proliferation. Using a specific FGFR1 tyrosine kinase inhibitor and conditioned medium with FGF2 immunoneutralised, we showed that the conditioned medium effects on endothelial cell network formation and proliferation was via FGF2-FGFR1 signaling.

To explore the signaling pathways activated in HUVECs by FGF2, following its release from epithelial cells in response to PGF_{2 α} -FP receptor activation, we used small molecule chemical inhibitors of intracellular signaling pathways. We found that conditioned medium enhanced endothelial cell network formation via FGFR1 and

ERK1/2 independently of PI3K and mTOR. This is in agreement with the observations of Kanda et al [32], who demonstrated in murine brain endothelial cells that FGF2 induced endothelial network formation is not dependent on activation of the mTOR pathway [32] and Sulpice et al [33] who showed that, in adrenal cortex capillary endothelial cells, ERK1/2 phosphorylation induced by recombinant FGF2 is not mediated via the PI3K pathway [33].

In contrast, we found that conditioned medium-induced endothelial cell proliferation was dependent on ERK1/2 signaling to mTOR since endothelial cell proliferation could be inhibited with the ERK1/2 kinase inhibitor PD98059 and rapamycin, but not the PI3K inhibitors wortmannin or LY294002. This is in agreement with previous studies showing that ERK1/2 inhibitor PD98059 can inhibit FGF2 induced angiogenesis [34] and HUVEC proliferation [35]. Our data indicate that proliferation and network formation are regulated by distinct signal transduction pathways which are integrated by ERK1/2 signaling.

In our studies we have shown that ERK1/2 is essential for both network formation and proliferation. ERK1/2 signaling is known to be a potent regulator of cell growth, differentiation and development [36]. We investigated conditioned medium signaling to ERK1/2 in HUVECs and found within our experimental paradigm that ERK1/2 was phosphorylated in a time dependent manner via the FGFR1 signaling to c-Src, independent of PI3K and mTOR. c-Src is a protein tyrosine kinase which coordinates a diverse spectrum of receptor-induced signaling to ERK1/2 via the phosphorylation of signaling intermediates such as Ras and Raf [37]. Src has been shown to be involved in

FGF-2 induced angiogenesis [38] and a recent study has shown that c-Src, Raf and ERK1/2 are essential for HUVEC lumen formation in vitro [39].

Following ERK1/2 activation, mTOR has been shown to be regulated via the tuberous sclerosis complex 1 and 2 (TSC1/2 also called Hamartin and Tuberin) [40, 41]. Phosphorylation of TSC2 by ERK1/2 results in its dissociation from TSC1 and its subsequent degradation via the ubiquitin pathway. This inactivates the inhibitory effect of TSC1/2 on the mTOR pathway [42] and allows cellular proliferation to proceed.

Over the past decade several reports have highlighted the importance of COX enzymes and prostaglandins in regulating vascular function indirectly. This may occur via the activation of ERK1/2 signaling and epithelial or stromal cell production of pro-angiogenic factors which act in a paracrine manner on endothelial cells [6, 43, 44]. This is in agreement with our observations here whereby FGF2 released by Ishikawa FPS cells in response to PGF_{2 α} enhanced the expression of COX-2 and FP receptor in endothelial cells via the FGF2-FGFR1-ERK1/2 pathway.

Additionally, prostaglandins have been shown to be secreted by endothelial cells and directly influence endothelial cell function via their receptors on endothelial cells [45-47]. We found that endothelial cells secrete elevated levels of PGF_{2 α} following activation by P CM of the FGF2-FGFR1-ERK1/2-mediated signaling pathway which upregulated COX-2 and endothelial FP receptor. Using a specific FP receptor antagonist and short hairpin RNA in an adenoviral delivery system for targeted ablation of endothelial FP receptor, we found that endothelial network formation was regulated by this secreted endothelial PGF_{2 α} . We also found an elevation in EP2 receptor expression in response to P CM and previous studies have shown that prostaglandin E₂ present in the

endothelial environment can enhance endothelial cell functions [45-47]. In our study we found no elevation in PGE₂ biosynthesis in response to P CM. Thus, since only prostaglandin F_{2 α} secretion was increased, we hypothesise that this is the prostaglandin predominantly responsible for the enhancement in HUVEC differentiation seen with P CM treatment.

As summarised in figure 8, our data show that PGF_{2 α} -FP receptor signaling in epithelial cells produces FGF2, which acts in a paracrine manner on endothelial FGFR1 to promote endothelial cell differentiation and proliferation via distinct intracellular mechanisms. We believe that these findings have relevance for endometrial pathologies, such as endometrial adenocarcinoma, which have aberrant expression of FP receptor and we propose a molecular mechanism whereby FP receptor may regulate vascular function [15, 19]. Furthermore our data suggest that targeted antagonism of epithelial FP receptor signaling, to reduce the production of growth factors, or endothelial FP receptor signaling, to prevent differentiation of endothelial cells, could provide an anti-angiogenic approach to reducing tumour vasculature and growth.

Funding

This study was supported by MRC core funding to HNJ (U.1276.00.004.00002.01)

Acknowledgements

The authors would like to thank Dr James Logie for helpful advice and technical assistance.

References

- [1] S. Westlake, N. Cooper, Cancer incidence and mortality: trends in the United Kingdom and constituent countries, 1993 to 2004, *Health Stat Q* (2008) 33-46.
- [2] A. Jemal, R. Siegel, E. Ward, Y. Hao, J. Xu, T. Murray, M.J. Thun, *Cancer statistics, 2008*, *CA Cancer J Clin* 58 (2008) 71-96.
- [3] R.H. Paulssen, B. Moe, H. Grønaas, A. Orbo, Gene expression in endometrial cancer cells (Ishikawa) after short time high dose exposure to progesterone, *Steroids* 73 (2008) 116-128.
- [4] E.D. Albrecht, G.W. Aberdeen, A.L. Niklaus, J.S. Babischkin, D.L. Suresch, G.J. Pepe, Acute temporal regulation of vascular endothelial growth/permeability factor expression and endothelial morphology in the baboon endometrium by ovarian steroids, *J Clin Endocrinol Metab* 88 (2003) 2844-2852.
- [5] A. Doll, M. Abal, M. Rigau, M. Monge, M. Gonzalez, S. Demajo, E. Colas, M. Llaurado, H. Alazzouzi, J. Planaguma, M.A. Lohmann, J. Garcia, S. Castellvi, J. Ramon y Cajal, A. Gil-Moreno, J. Xercavins, F. Alameda, J. Reventos, Novel molecular profiles of endometrial cancer-new light through old windows, *J Steroid Biochem Mol Biol* 108 (2008) 221-229.

[6] H.N. Jabbour, K.J. Sales, O.P. Smith, S. Battersby, S.C. Boddy, Prostaglandin receptors are mediators of vascular function in endometrial pathologies, *Mol Cell Endocrinol* 252 (2006) 191-200.

[7] B.J. Tong, J. Tan, L. Tajeda, S.K. Das, J.A. Chapman, R.N. DuBois, S.K. Dey, Heightened expression of cyclooxygenase-2 and peroxisome proliferator-activated receptor-delta in human endometrial adenocarcinoma, *Neoplasia* 2 (2000) 483-490.

[8] S. Tsuji, S. Kawano, H. Sawaoka, Y. Takei, I. Kobayashi, K. Nagano, H. Fusamoto, T. Kamada, Evidences for involvement of cyclooxygenase-2 in proliferation of two gastrointestinal cancer cell lines, *Prostaglandins Leukot Essent Fatty Acids* 55 (1996) 179-183.

[9] M. Tsujii, R.N. DuBois, Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2, *Cell* 83 (1995) 493-501.

[10] M. Tsujii, S. Kawano, S. Tsuji, H. Sawaoka, M. Hori, R.N. DuBois, Cyclooxygenase regulates angiogenesis induced by colon cancer cells, *Cell* 93 (1998) 705-716.

[11] J. Folkman, Tumor angiogenesis: therapeutic implications, *N Engl J Med* 285 (1971) 1182-1186.

[12] G. Bergers, L.E. Benjamin, Tumorigenesis and the angiogenic switch, *Nat Rev Cancer* 3 (2003) 401-410.

- [13] D.H. Ausprunk, J. Folkman, Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis, *Microvasc Res* 14 (1977) 53-65.
- [14] K.J. Sales, S.C. Boddy, A.R. Williams, R.A. Anderson, H.N. Jabbour, F-prostanoid receptor regulation of fibroblast growth factor 2 signaling in endometrial adenocarcinoma cells, *Endocrinology* 148 (2007) 3635-3644.
- [15] K.J. Sales, T. List, S.C. Boddy, A.R. Williams, R.A. Anderson, Z. Naor, H.N. Jabbour, A novel angiogenic role for prostaglandin F2alpha-FP receptor interaction in human endometrial adenocarcinomas, *Cancer Res* 65 (2005) 7707-7716.
- [16] G. Soufla, S. Sifakis, D.A. Spandidos, FGF2 transcript levels are positively correlated with EGF and IGF-1 in the malignant endometrium, *Cancer Lett* 259 (2008) 146-155.
- [17] T. Danielsen, E.K. Rofstad, VEGF, bFGF and EGF in the angiogenesis of human melanoma xenografts, *Int J Cancer* 76 (1998) 836-841.
- [18] E.K. Rofstad, E.F. Halsor, Vascular endothelial growth factor, interleukin 8, platelet-derived endothelial cell growth factor, and basic fibroblast growth factor promote angiogenesis and metastasis in human melanoma xenografts, *Cancer Res* 60 (2000) 4932-4938.
- [19] K.J. Sales, S.A. Milne, A.R. Williams, R.A. Anderson, H.N. Jabbour, Expression, localization, and signaling of prostaglandin F2 alpha receptor in human endometrial adenocarcinoma: regulation of proliferation by activation of the

epidermal growth factor receptor and mitogen-activated protein kinase signaling pathways, *J Clin Endocrinol Metab* 89 (2004) 986-993.

[20] K.J. Sales, S. Battersby, A.R. Williams, R.A. Anderson, H.N. Jabbour, Prostaglandin E2 mediates phosphorylation and down-regulation of the tuberous sclerosis-2 tumor suppressor (tuberin) in human endometrial adenocarcinoma cells via the Akt signaling pathway, *J Clin Endocrinol Metab* 89 (2004) 6112-6118.

[21] K.J. Sales, A.A. Katz, B. Howard, R.P. Soeters, R.P. Millar, H.N. Jabbour, Cyclooxygenase-1 is up-regulated in cervical carcinomas: autocrine/paracrine regulation of cyclooxygenase-2, prostaglandin E receptors, and angiogenic factors by cyclooxygenase-1, *Cancer Res* 62 (2002) 424-432.

[22] S. Battersby, K.J. Sales, A.R. Williams, R.A. Anderson, S. Gardner, H.N. Jabbour, Seminal plasma and prostaglandin E2 up-regulate fibroblast growth factor 2 expression in endometrial adenocarcinoma cells via E-series prostanoid-2 receptor-mediated transactivation of the epidermal growth factor receptor and extracellular signal-regulated kinase pathway, *Hum Reprod* 22 (2007) 36-44.

[23] M.A. Ledingham, F.C. Denison, R.W. Kelly, A. Young, J.E. Norman, Nitric oxide donors stimulate prostaglandin F(2alpha) and inhibit thromboxane B(2) production in the human cervix during the first trimester of pregnancy, *Mol Hum Reprod* 5 (1999) 973-982.

[24] F.C. Denison, A.A. Calder, R.W. Kelly, The action of prostaglandin E2 on the human cervix: stimulation of interleukin 8 and inhibition of secretory leukocyte protease inhibitor, *Am J Obstet Gynecol* 180 (1999) 614-620.

[25] M. Majima, I. Hayashi, M. Muramatsu, J. Katada, S. Yamashina, M. Katori, Cyclo-oxygenase-2 enhances basic fibroblast growth factor-induced angiogenesis through induction of vascular endothelial growth factor in rat sponge implants, Br J Pharmacol 130 (2000) 641-649.

[26] P. Auguste, S. Javerzat, A. Bikfalvi, Regulation of vascular development by fibroblast growth factors, Cell Tissue Res 314 (2003) 157-166.

[27] M.A. Konerding, E. Fait, C. Dimitropoulou, W. Malkusch, C. Ferri, R. Giavazzi, D. Coltrini, M. Presta, Impact of fibroblast growth factor-2 on tumor microvascular architecture. A tridimensional morphometric study, Am J Pathol 152 (1998) 1607-1616.

[28] Y. Wang, D. Becker, Antisense targeting of basic fibroblast growth factor and fibroblast growth factor receptor-1 in human melanomas blocks intratumoral angiogenesis and tumor growth, Nat Med 3 (1997) 887-893.

[29] J. Folkman, C. Haudenschild, Angiogenesis in vitro, Nature 288 (1980) 551-556.

[30] D. Hanahan, J. Folkman, Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis, Cell 86 (1996) 353-364.

[31] R.H. Adams, K. Alitalo, Molecular regulation of angiogenesis and lymphangiogenesis, Nat Rev Mol Cell Biol 8 (2007) 464-478.

[32] S. Kanda, M.N. Hodgkin, R.J. Woodfield, M.J. Wakelam, G. Thomas, L. Claesson-Welsh, Phosphatidylinositol 3'-kinase-independent p70 S6 kinase activation by fibroblast growth factor receptor-1 is important for proliferation but not differentiation of endothelial cells, J Biol Chem 272 (1997) 23347-23353.

[33] E. Sulpice, M. Bryckaert, J. Lacour, J.O. Contreres, G. Tobelem, Platelet factor 4 inhibits FGF2-induced endothelial cell proliferation via the extracellular signal-regulated kinase pathway but not by the phosphatidylinositol 3-kinase pathway, *Blood* 100 (2002) 3087-3094.

[34] B.P. Eliceiri, R. Klemke, S. Stromblad, D.A. Cheresh, Integrin alphavbeta3 requirement for sustained mitogen-activated protein kinase activity during angiogenesis, *J Cell Biol* 140 (1998) 1255-1263.

[35] L.W. Wu, L.D. Mayo, J.D. Dunbar, K.M. Kessler, M.R. Baerwald, E.A. Jaffe, D. Wang, R.S. Warren, D.B. Donner, Utilization of distinct signaling pathways by receptors for vascular endothelial cell growth factor and other mitogens in the induction of endothelial cell proliferation, *J Biol Chem* 275 (2000) 5096-5103.

[36] T.S. Lewis, P.S. Shapiro, N.G. Ahn, Signal transduction through MAP kinase cascades, *Adv Cancer Res* 74 (1998) 49-139.

[37] S.M. Thomas, J.S. Brugge, Cellular functions regulated by Src family kinases, *Annu Rev Cell Dev Biol* 13 (1997) 513-609.

[38] W.W. Kilar斯基, N. Jura, P. Gerwinski, Inactivation of Src family kinases inhibits angiogenesis in vivo: implications for a mechanism involving organization of the actin cytoskeleton, *Exp Cell Res* 291 (2003) 70-82.

[39] W. Koh, K. Sachidanandam, A.N. Stratman, A. Sacharidou, A.M. Mayo, E.A. Murphy, D.A. Cheresh, G.E. Davis, Formation of endothelial lumens requires a coordinated PKC ϵ -, Src-, Pak- and Raf-kinase-dependent signaling cascade downstream of Cdc42 activation, *J Cell Sci* 122 (2009) 1812-1822.

[40] D.A. Guertin, D.M. Sabatini, Defining the role of mTOR in cancer, *Cancer Cell* 12 (2007) 9-22.

[41] D.F. Lee, M.C. Hung, All roads lead to mTOR: integrating inflammation and tumor angiogenesis, *Cell Cycle* 6 (2007) 3011-3014.

[42] E. Drakos, G.Z. Rassidakis, L.J. Medeiros, Mammalian target of rapamycin (mTOR) pathway signalling in lymphomas, *Expert Rev Mol Med* 10 (2008) e4.

[43] D. Wang, H. Wang, J. Brown, T. Daikoku, W. Ning, Q. Shi, A. Richmond, R. Strieter, S.K. Dey, R.N. DuBois, CXCL1 induced by prostaglandin E2 promotes angiogenesis in colorectal cancer, *J Exp Med* 203 (2006) 941-951.

[44] H.N. Jabbour, K.J. Sales, Prostaglandin receptor signalling and function in human endometrial pathology, *Trends Endocrinol Metab* 15 (2004) 398-404.

[45] F. Finetti, R. Solito, L. Morbidelli, A. Giachetti, M. Ziche, S. Donnini, Prostaglandin E2 regulates angiogenesis via activation of fibroblast growth factor receptor-1, *J Biol Chem* 283 (2008) 2139-2146.

[46] R. Rao, R. Redha, I. Macias-Perez, Y. Su, C. Hao, R. Zent, M.D. Breyer, A. Pozzi, Prostaglandin E2-EP4 receptor promotes endothelial cell migration via ERK activation and angiogenesis in vivo, *J Biol Chem* 282 (2007) 16959-16968.

[47] K. Tamura, T. Sakurai, H. Kogo, Relationship between prostaglandin E2 and vascular endothelial growth factor (VEGF) in angiogenesis in human vascular endothelial cells, *Vascul Pharmacol* 44 (2006) 411-416.

Figures

Fig. 1. The effect of PGF conditioned medium on endothelial network formation and proliferation. **(A)** Protein concentration of FGF2 (pg/ml) present in conditioned medium (CM) from Ishikawa FPS cells treated with vehicle (V), 100nM PGF_{2α} (P). FGF2 was immunoneutralised from (P) using a specific FGF2 antibody (P+FGF2Ab) as described in the methods. **(B)** Endothelial network formation and **(C)** proliferation was investigated using HUVECs stimulated with V CM (V), P CM (P) or P CM in the presence of FGFR1 tyrosine kinase inhibitor (P+ SU4984) or FGF2 immunoneutralised CM (P + FGF2Ab). (* is significantly different from V CM, ** is significantly different from *; P<0.05). Data are represented as percentage increase compared to V CM and presented as mean ± SEM.

Fig. 2. Endothelial network formation and proliferation is mediated via the ERK1/2 pathway. **(A)** Endothelial network formation and **(B)** proliferation in HUVECs treated with V CM or PGF CM in the absence/presence of inhibitors of ERK1/2 (PD98059), mTOR (Rapamycin) or PI3K (Wortmannin or LY294002). **(C)** Endothelial network formation and **(D)** proliferation in HUVECs treated with vehicle or recombinant FGF2 protein (50ng/ul) in the absence/presence of Rapamycin. (* represents statistical significance P<0.05; ** is significantly different from *; P<0.05). Data are represented as percentage increase compared to V CM and presented as mean ± SEM.

Fig. 3. Conditioned medium induces expression of COX-2, FP receptor and EP2 receptor. Expression of (A) COX-1, (B) COX-2, (C) FP receptor and (D) EP2 receptor mRNA in HUVECs incubated with V CM or P CM for 1,2,3,4,6,16 and 24 hours as determined by quantitative RT-PCR analysis. (* represents statistical significance P<0.05) Data are represented as mean \pm SEM.

Fig. 4. COX-2, FP receptor and EP2 receptor expression is regulated by FGFR1 and ERK1/2. Expression of (A) COX-2, (B) FP receptor and (C) EP2 receptor mRNA in HUVECs incubated with V CM or P CM for 3 hours in the absence/presence of FGFR1 tyrosine kinase inhibitor SU4984 or ERK1/2 inhibitor PD98059 as determined by quantitative RT-PCR analysis. (* represents statistical significance P<0.05, ** is significantly different from *; P<0.05). Data are represented as mean \pm SEM.

Fig. 5. The effect of conditioned medium on ERK1/2 signaling in HUVECs. (A) HUVECs were treated with V CM or P CM for 5,10,15,20 and 30 minutes and (B) HUVECs were treated with V CM or P CM for 10 min in the absence/presence of SU4984, PP2, PD98059 or LY294002 and immunoblot analysis was carried out with antibodies against phosphorylated ERK1/2 (top panel) and total ERK1/2 (bottom panel). A representative western blot is displayed with a graph of semi-quantitative analysis of ERK phosphorylation determined as described in Materials and Methods. (* represents statistical significance, ** is significantly different from *; P<0.05). Data are represented as mean \pm SEM.

Fig. 6. PGF_{2 α} is produced in endothelial cells in response to conditioned medium. HUVECs were incubated with V CM or P CM for 0, 3, 6 and 16 hours and thereafter conditioned medium from HUVECs was collected and used for PGE₂ (A) and PGF_{2 α} (B) ELISA analysis as described in the methods. (* represents statistical significance P<0.05)

Data are represented as mean \pm SEM.

Fig. 7. Endothelial network formation is regulated by endothelial PGF_{2 α} -FP receptor interaction. (A) Endothelial network formation and (B) proliferation was investigated with HUVECs treated with V CM, P CM, vehicle or 1 μ M PGF_{2 α} in the absence or presence of specific FP receptor antagonist AL8810. (C) HUVECs were infected with scrambled adenovirus (Scr) or short hairpin adenovirus targeted against the FP receptor (sh306 or sh478) and FP receptor knock-down was determined by quantitative RT-PCR. HUVECs were infected with scrambled adenovirus (Scr), sh306 or sh478 and treated with V CM or P CM and (D) endothelial network formation and (E) proliferation was determined as described in the methods. (* represents significance P<0.05; ** is significantly different from *; P<0.05). Data are represented as mean \pm SEM.

Fig. 8. A schematic representation of PGF_{2 α} -FP receptor signaling which differentially regulates endothelial network formation and proliferation via FGF2-FGFR1 pathway. PGF_{2 α} -FP receptor signaling in epithelial cells promotes the release of FGF2 into culture medium (conditioned medium). Conditioned medium treatment of HUVECs promotes the activation of ERK1/2 signaling via the FGF2-FGFR1. ERK1/2 activates divergent signaling pathways to endothelial proliferation and network formation. Endothelial proliferation is activated via mTOR. Endothelial network formation is activated via the induction of COX-2 and endothelial FP receptor and biosynthesis and release of PGF_{2 α} . Endothelial PGF_{2 α} subsequently acts on endothelial FP receptors to promote network formation (cellular differentiation).

Fig. 1

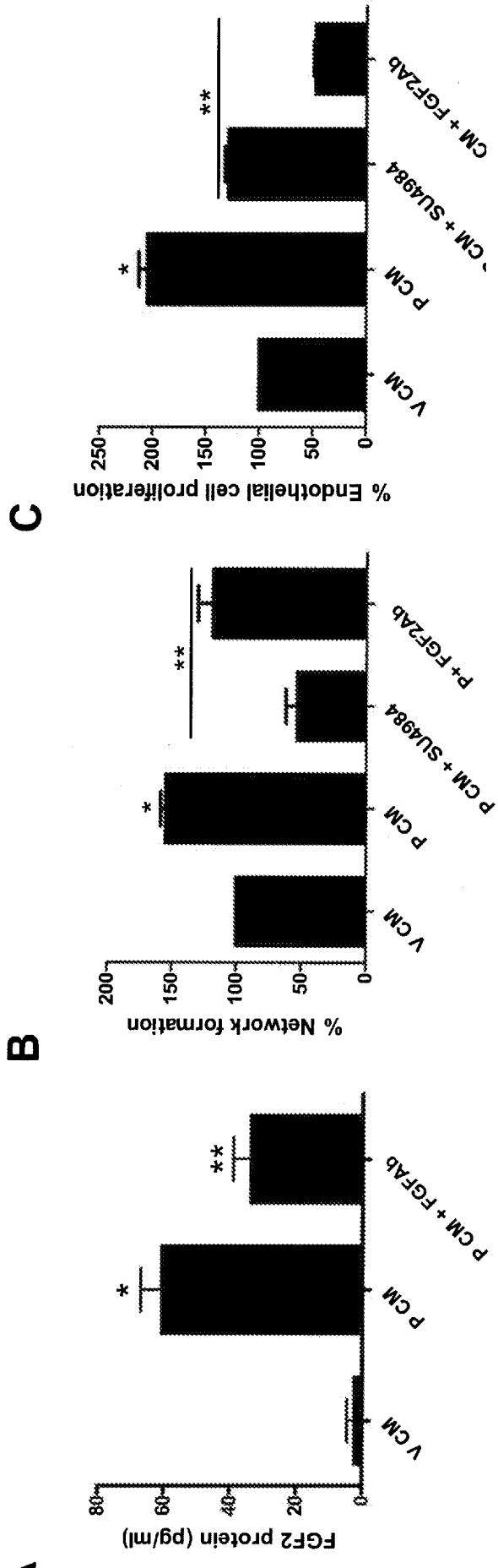


Fig. 2

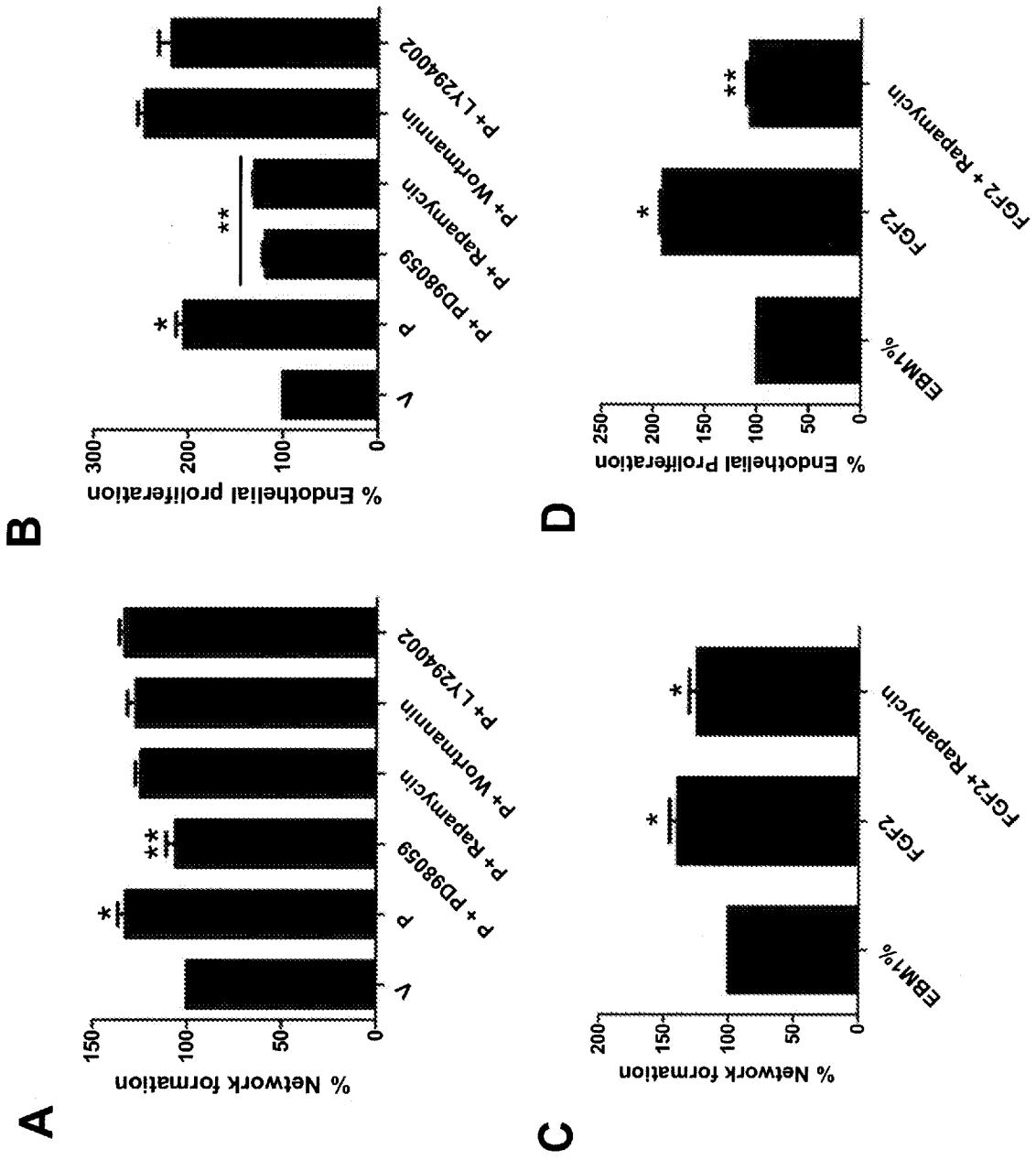


Fig. 3

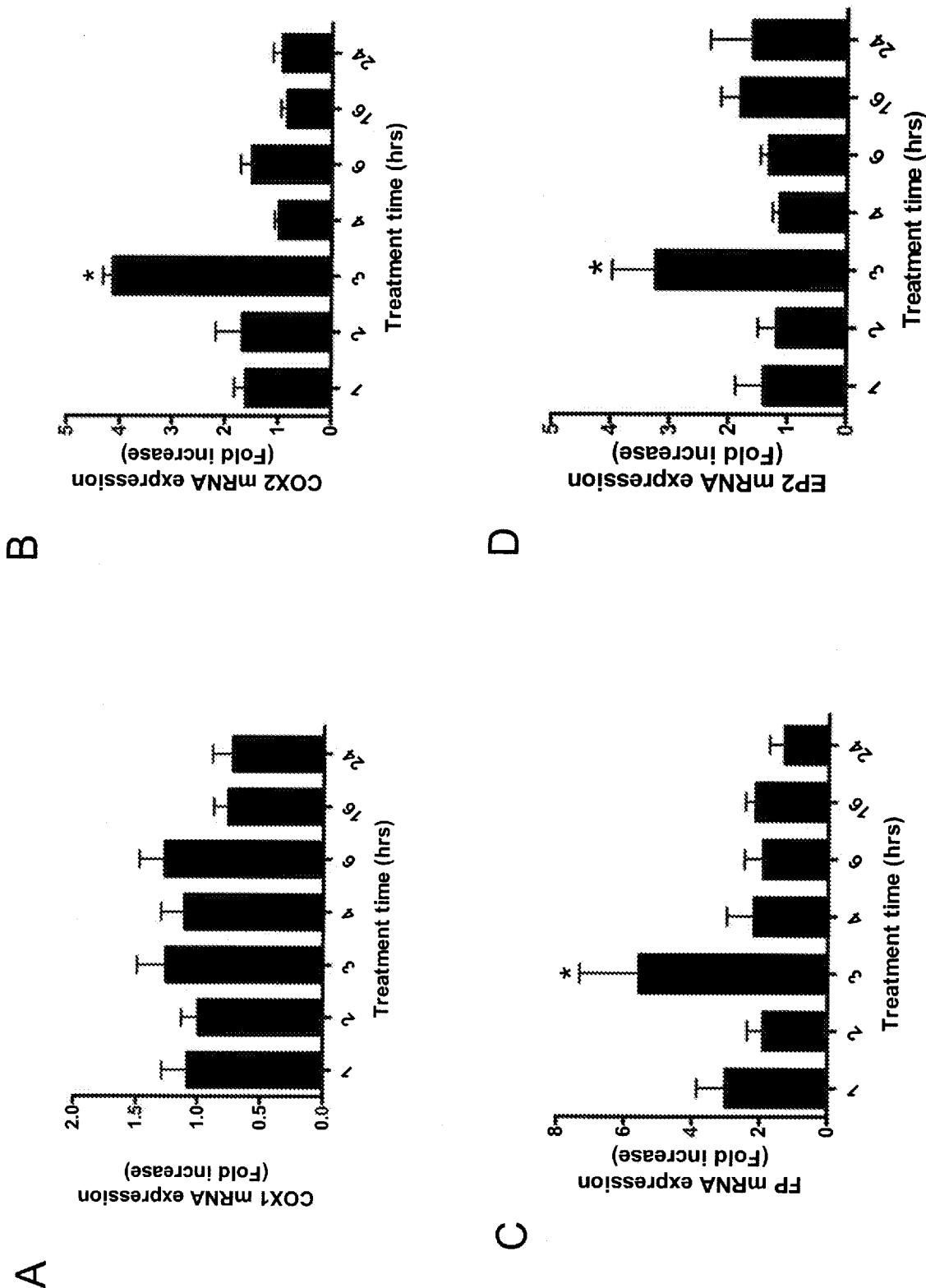


Fig. 4

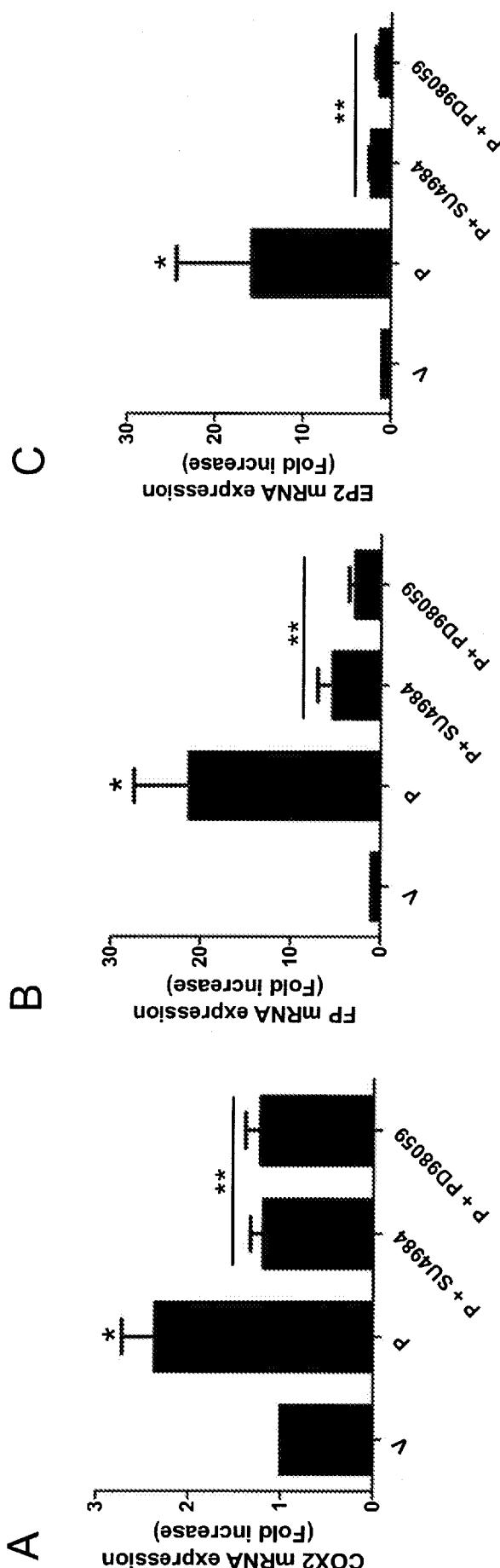


Fig. 5

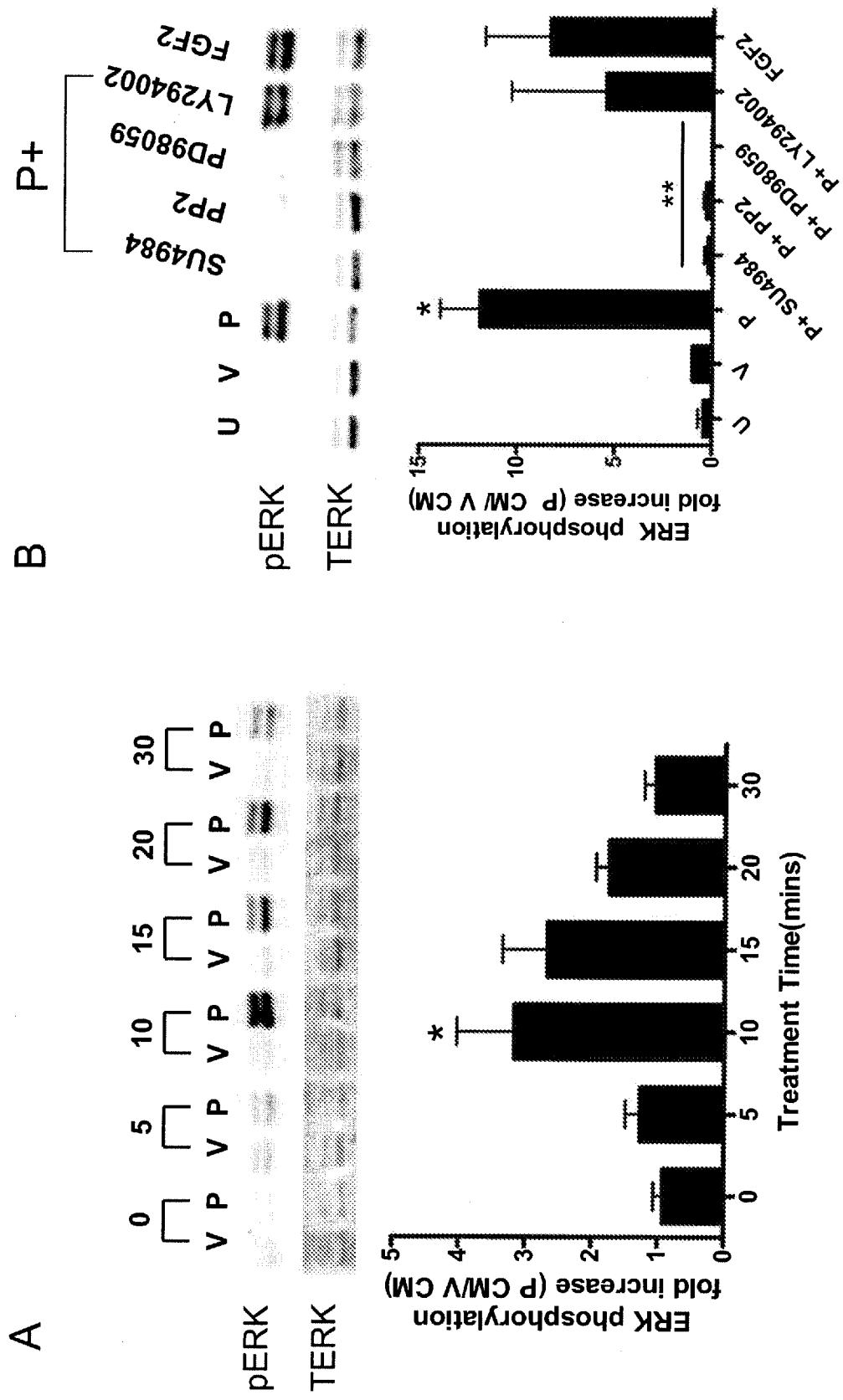


Fig. 6

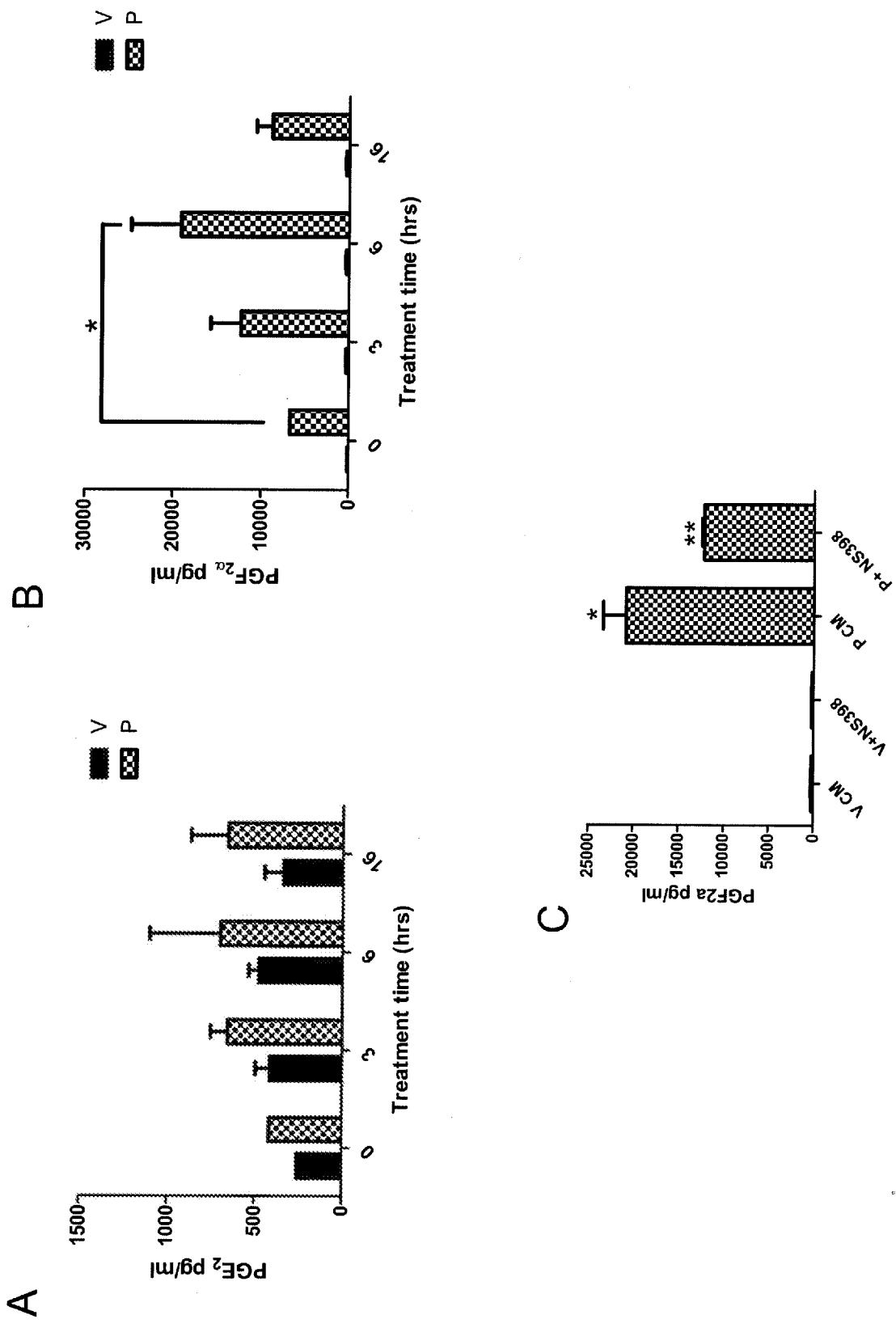


Fig. 7

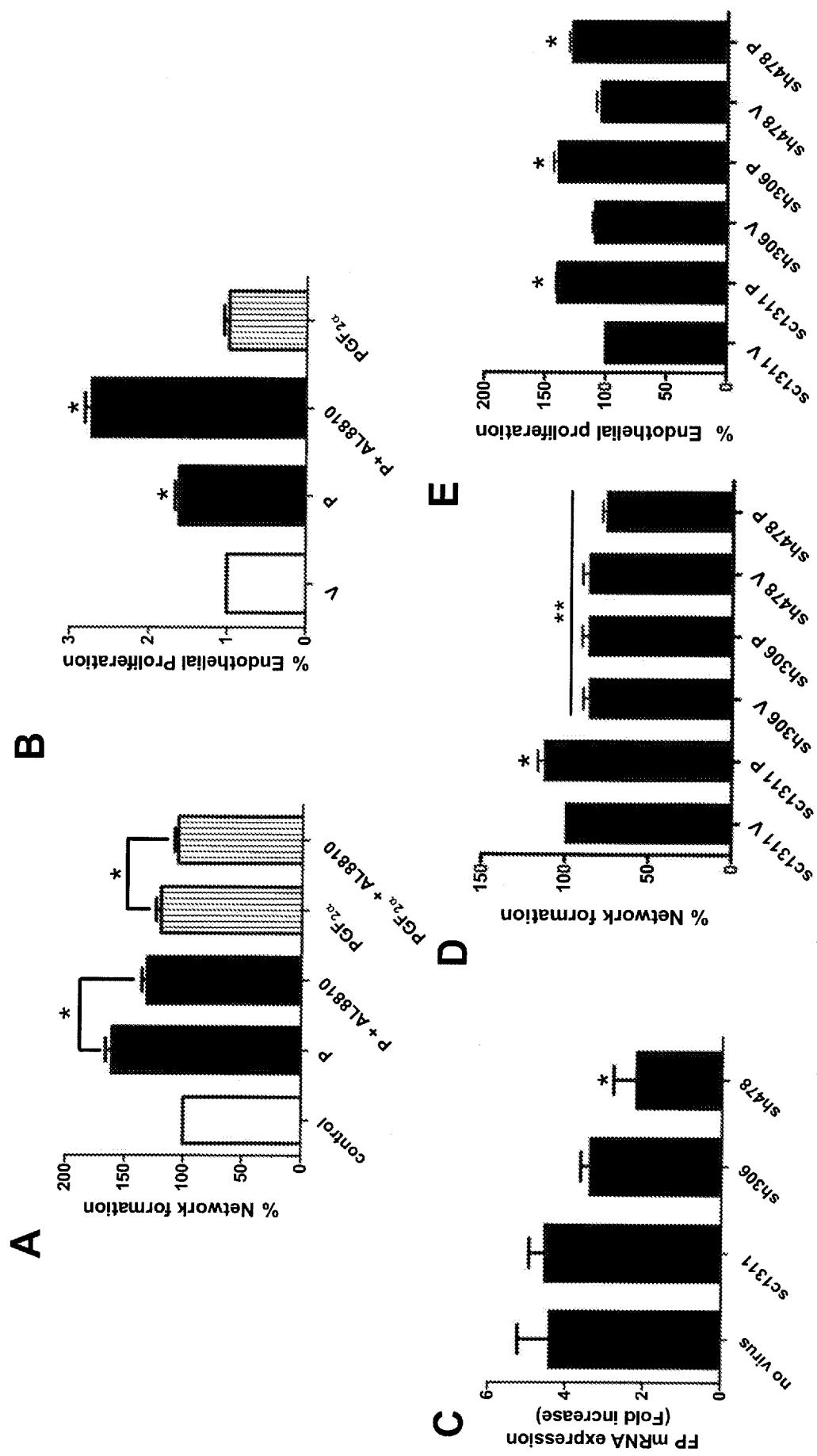
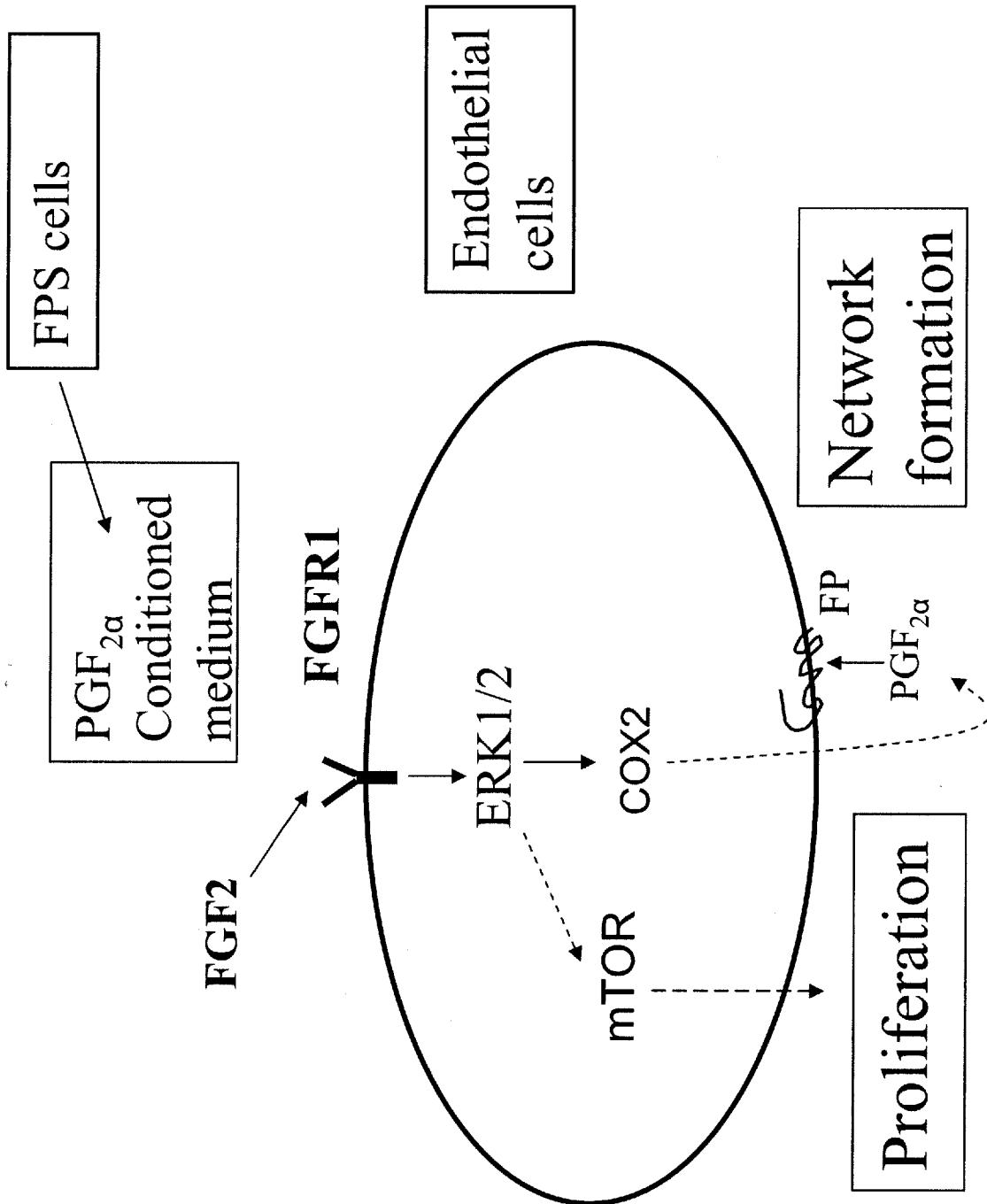


Fig. 8



IN THE MATTER of US Patent Application
Serial No 10/511,480

Annex 2

Wallace *et al* (2009) *Cancer Res* 69(14), 5726-5733

Signed

LENNY JEFFREY

Dated

14th August 2009

Prostaglandin $F_{2\alpha}$ -F-Prostanoid Receptor Signaling Promotes Neutrophil Chemotaxis via Chemokine (C-X-C Motif) Ligand 1 in Endometrial Adenocarcinoma

Alison E. Wallace,¹ Kurt J. Sales,¹ Roberto D. Catalano,¹ Richard A. Anderson,^{1,2} Alistair R.W. Williams,³ Martin R. Wilson,¹ Jurgen Schwarze,⁴ Hongwei Wang,⁴ Adriano G. Rossi,⁴ and Henry N. Jabbour¹

¹Medical Research Council Human Reproductive Sciences Unit, Departments of ²Reproductive and Developmental Sciences and ³Pathology, and ⁴Centre for Inflammation Research, The Queen's Medical Research Institute, Edinburgh, United Kingdom

Abstract

The prostaglandin $F_{2\alpha}$ (PGF $F_{2\alpha}$) receptor (FP) is elevated in endometrial adenocarcinoma. This study found that PGF $F_{2\alpha}$ signaling via FP regulates expression of chemokine (C-X-C motif) ligand 1 (CXCL1) in endometrial adenocarcinoma cells. Expression of CXCL1 and its receptor, CXCR2, are elevated in cancer tissue compared with normal endometrium and localized to glandular epithelium, endothelium, and stroma. Treatment of Ishikawa cells stably transfected with the FP receptor (FPS cells) with 100 nmol/L PGF $F_{2\alpha}$ increased CXCL1 promoter activity, mRNA, and protein expression, and these effects were abolished by cotreatment of cells with FP antagonist or chemical inhibitors of Gq, epidermal growth factor receptor, and extracellular signal-regulated kinase. Similarly, CXCL1 was elevated in response to 100 nmol/L PGF $F_{2\alpha}$ in endometrial adenocarcinoma explant tissue. CXCL1 is a potent neutrophil chemoattractant. The expression of CXCR2 colocalized to neutrophils in endometrial adenocarcinoma and increased neutrophils were present in endometrial adenocarcinoma compared with normal endometrium. Conditioned media from PGF $F_{2\alpha}$ -treated FPS cells stimulated neutrophil chemotaxis, which could be abolished by CXCL1 protein immunoneutralization of the conditioned media or antagonism of CXCR2. Finally, xenograft tumors in nude mice arising from inoculation with FPS cells showed increased neutrophil infiltration compared with tumors arising from wild-type cells or following treatment of mice bearing FPS tumors with CXCL1-neutralizing antibody. In conclusion, our results show a novel PGF $F_{2\alpha}$ -FP pathway that may regulate the inflammatory microenvironment in endometrial adenocarcinoma via neutrophil chemotaxis. [Cancer Res 2009;69(14):5726–33]

Introduction

Endometrial adenocarcinoma is the most common gynecological malignancy in Western countries, affecting mainly postmenopausal women with a frequency of 15 to 20 per 100,000 women per year (1). Overexpression of the cyclooxygenase (COX) enzymes and prostaglandins has been shown in endometrial adenocarcinoma as

well as a number of other cancer types and gynecological pathologies (2, 3).

In the reproductive tract, the most commonly synthesized prostaglandins are the E- and F-series prostanoids (4). These are synthesized from arachidonic acid by COX enzymes and prostaglandin synthases, and are then transported out of the cell by a prostaglandin transporter (5) to act in an autocrine/paracrine manner on G-protein-coupled receptors. The G-protein-coupled receptor for PGF $F_{2\alpha}$ (FP) is a Gq coupled receptor, which upon activation leads to release of inositol-1,4,5-trisphosphate and diacylglycerol (6). Recently, we have shown a role for FP in endometrial adenocarcinoma, with evidence for elevated PGF $F_{2\alpha}$ -FP signaling up-regulating angiogenic and tumorigenic genes including COX-2 (7), FGF2 (8), and vascular endothelial growth factor (VEGF; ref. 9), and increasing proliferation and migration of neoplastic epithelial cells (10–12).

Chemokine (C-X-C motif) ligand 1 (CXCL1, also known as growth-regulated oncogene α) has angiogenic, chemoattractant, and inflammatory activities (13). A link between prostaglandins and CXCL1 has been shown, as prostaglandin E₂ signaling induces CXCL1 expression in colorectal cancer cell lines (14). CXCL1 is up-regulated in melanoma (15, 16), colorectal (17, 18), and prostate cancer (19) and binds to the CXCR2 receptor (20) to promote the recruitment of neutrophils to sites of inflammation (21). Although the role of neutrophils in cancer is unclear, recent evidence suggests that they may promote tissue remodeling by production of proteases including matrix metalloproteinase (MMP)-9 (22) and angiogenic factors such as VEGF (23), in addition to their classic role as the first line of defense against invading pathogens (24).

In this study, we used a chemokine antibody array to identify CXCL1 as a target gene of PGF $F_{2\alpha}$. We investigated the expression, localization, and regulation of CXCL1 expression mediated via PGF $F_{2\alpha}$ -FP signaling in endometrial adenocarcinoma and its downstream regulation of neutrophil influx into endometrial tumors *in vitro* and in endometrial tumor xenografts *in vivo*.

Materials and Methods

Reagents. Indomethacin, PBS, bovine serum albumin (BSA), AL8810, Tris, reagent, and PGF $F_{2\alpha}$ were purchased from Sigma Co. PD98059, AG1478, Cyclosporin A, and 4C3MQ were purchased from Calbiochem. CXCR2 and Gr-1 (a murine neutrophil marker) antibodies were purchased from R&D systems, and CXCL1 and neutrophil elastase antibodies from Santa Cruz Biotechnology and DAKO, respectively. FITC-CD11b, PE-GR-1, and Cy5-CD11c antibodies were obtained from eBioscience. The chemokine antibody array was purchased from RayBiotech.

Patients and tissue collection. Endometrial adenocarcinoma tissue and normal tissue was obtained as detailed in our prior studies (9, 10). Cancer

Requests for reprints: Henry Jabbour, Medical Research Council Human Reproductive Sciences Unit, The Queen's Medical Research Institute, Edinburgh EH16 4TJ, United Kingdom. Phone: 44-1312426220; Fax: 44-1312426231; E-mail: H.Jabbour@hrs.u.mrc.ac.uk.

©2009 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-09-0390

patients were prediagnosed to have adenocarcinoma of the uterus, and diagnosis was confirmed histologically in all cases. Normal endometrial tissue was collected from women undergoing surgery for minor gynecologic procedures with no underlying endometrial pathology. Ethical approval was obtained from Lothian Research Ethics Committee, and written informed consent was obtained from all subjects before tissue collection.

Cell lines, culture, and treatments. Wild-type (WT) Ishikawa cells and Ishikawa cells engineered to stably express the full-length human FP receptor to the levels observed in endometrial adenocarcinomas, called FPS cells, were cultured as described previously (9). Transient transfections were performed using Superfect (Qiagen) as per the manufacturer's protocol. The optimal concentrations of all chemical inhibitors and antibodies were determined empirically by titration using the manufacturer's guidelines as described in our previous studies (25). Cell viability was determined for each inhibitor using the CellTiter 96 AQueous One Solution assay (Promega) as described in our previous studies (10, 25). Within the time frame of incubation in this study, the inhibitors had no adverse effect on cell viability, at the concentration used. Cells were treated with vehicle, inhibitor alone, or 100 nmol/L PGF_{2α} alone or in the presence of YM254890 (1 μmol/L), AL8810 (50 μmol/L), 43CMQ (1 μmol/L), AG1478 (200 nmol/L), or Cyclosporine A (CsA; 1 μmol/L) for the time indicated.

Chemokine antibody array. Conditioned medium (CM) was prepared as described (8). Briefly, FPS cells were stimulated with vehicle or 100 nmol/L PGF_{2α} for 24 h. The CM (V-CM or P-CM, respectively) was analyzed for cytokine expression using the RayBio Human Cytokine Antibody Array 3 kit, following manufacturer's protocol.

CXCL1 luciferase reporter assays. The CXCL1 reporter plasmid consisting of the CXCL1 promoter fused to the firefly luciferase reporter (as described in ref. 26) was kindly supplied by Professor Ann Richmond (Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, Tennessee). The CXCL1 promoter firefly luciferase reporter was transfected into FPS cells with pRL-TK (containing the renilla luciferase coding sequence; Promega) as an internal control. FPS cells were cotransfected with control vector (pcDNA3.0) or vector encoding a dominant negative (DN) isoform of EGFR, Ras, MAP/ERK kinase (MEK), or NFAT (kindly supplied by Professor Zvi Naor, Tel Aviv University, Israel). The DN constructs have been previously characterized and described (27, 28). Cells were stimulated with vehicle or 100 nmol/L PGF_{2α} for the time indicated in the figure legend. The activity of firefly and renilla was determined using the dual luciferase assay kit (Promega) and total luciferase activity determined relative to the internal renilla control. Data are expressed as fold increase in luciferase activity compared with vehicle-treated cells and are presented as mean ± SE from at least three independent experiments.

Taqman quantitative reverse transcription-PCR. CXCL1 and CXCR2 expression in endometrial tissues and FPS cells was measured by quantitative reverse transcription-PCR analysis as described previously (1, 2). FPS cells were treated with vehicle, 100 nmol/L PGF_{2α} alone or in the presence of inhibitor, or vehicle and inhibitor alone for 8 h. RNA samples were then extracted using Tri-reagent following manufacturer's guidelines. RNA samples were reverse transcribed and RT-PCR performed as described previously (1, 2) using sequence specific primers and probes: CXCL1 forward, 5'-GTT TTC AAA TGT TCT CCA GTC ATT ATG-3'; reverse, 5'-CCG CCA GCC TCT ATC ACA GT-3'; probe, 5'-TTC TGA GGA GCC TGC AAC ATG CCA-3'; CXCR2 forward, 5'-TGC TCT TCT GGA GGT GTC CTA CA-3'; reverse, 5'-AGA TCT TCA CCT TTC CAG AAA TCT T-3'; probe, 5'-CCC AGC GAC CCA GTC AGG ATT TAA-3'. Primers and data were analyzed and processed using Sequence Detector v1.6.3 (Applied Biosystems). Expression of analyzed genes was normalized to RNA loading for each sample using the 18S rRNA as an internal standard. Results are expressed as fold increase above cells treated with vehicle and inhibitor. Data are presented as mean ± SE from at least three independent experiments.

CXCL1 ELISA. CXCL1 protein secretion by FPS cells in the culture media was measured by the Human CXCL1 Quantikine ELISA kit (R&D systems). FPS cells were treated as described above for mRNA, for time indicated in the figure legend. The ELISA was then carried out according to manufacturer's instructions. Absorbance of wells was determined by

spectrophotometry at 450 nm. Data are presented as mean ± SE from at least three independent experiments.

Immunohistochemical analysis. Expression of CXCL1, neutrophil elastase, CXCR2, and Gr-1 was localized in endometrial tissue and xenografts by immunohistochemistry using standard techniques as described previously (1, 2). Briefly, following antigen retrieval, sections were blocked in 5% normal rabbit serum (CXCL1 and Gr-1) or normal goat serum (CXCR2 and neutrophil elastase) diluted in TBS with 5% BSA. Subsequently, tissue sections were incubated with goat anti-human CXCL1 polyclonal antibody (2 μg/mL), mouse anti-human CXCR2 (5 μg/mL), mouse anti-human neutrophil elastase monoclonal antibody (2 μg/mL), or rat anti-mouse Gr-1 (5 μg/mL) overnight at 4°C. Control sections included the following: no primary antibody, nonimmune goat, mouse and rat IgG, or CXCL1 antibody preabsorbed with blocking peptide (20 μg/mL; Santa Cruz Biotechnology). Subsequently, sections were incubated with rabbit anti-goat/rat biotinylated or goat anti-mouse biotinylated antibodies (DAKO), followed by streptavidin-horse radish peroxidase complex (DAKO). Color reaction was developed with 3,3' diaminobenzidine (DAKO). Sections were counterstained in hematoxylin. Images were obtained on a PROVIS microscope at ×200 or ×400 magnification (Olympus Optical) using Canon EOS image capture software (Canon). The number of neutrophils was quantified using neutrophil elastase staining and standard stereological techniques. Briefly, each section was examined using ×40 plan apo objective from a BH2 microscope (Olympus) fitted with an automatic stage (Prior Scientific Instruments Ltd.) using a video camera (HV-C20; Hitachi) and analyzed with Image-ProPlus 4.5.1 software with a Stereology 5.0 plug-in (Media Cybernetics). A total of 40 randomized fields of view were examined and counted ($n = 7$ normal endometrium; $n = 30$ carcinoma), and data are expressed as mean number of cells per mm² of tumor examined.

Immunofluorescence microscopy. CXCR2 expression was colocalized with neutrophil elastase by immunofluorescence microscopy as described previously (8, 29). Briefly, sections were blocked in 5% normal goat serum diluted in PBS with 5% BSA before incubation with mouse anti-neutrophil elastase (1 μg/mL). Following overnight incubation at 4°C, sections were incubated with goat anti-mouse biotinylated Fab, then tyramide signal amplification kit (TSA Fluorescein System; 1:50 dilution; Perkin-Elmer). Sections were then microwaved in 0.01 M citrate buffer for 30 min and endogenous peroxidase blocked using hydrogen peroxide. Nonspecific binding was blocked with 5% normal goat serum and sections were incubated with mouse anti-CXCR2 antibody (1 μg/mL) at 4°C overnight. Sections were again incubated with goat anti-mouse biotinylated Fab and tyramide signal amplification kit. Nuclei were stained using ToPro (Molecular Probes). Fluorescent images were visualized and photographed using a Carl Zeiss laser scanning microscope LSM510 (×400 objective; Jena).

Neutrophil chemotaxis assay. Neutrophil chemotaxis was analyzed using transwell inserts (5-μm pore size; Corning Costar). Neutrophils were purified as previously described (30) and resuspended in serum free media. Cells (750,000) were added to the top chamber of the transwell insert and 600 μL of V-CM or P-CM was added to the bottom chamber. Serum-free media alone or with 50,000 pg/mL CXCL1 were added as negative and positive controls, respectively. Cells were incubated at 37°C in a 5% CO₂ atmosphere for 1 h and the plate was gently tapped to dislodge cells adhered to the underside of the membrane. Cells in the bottom chamber were collected and counted at least six times using a haemocytometer. Data are expressed as mean ± SE from at least three independent experiments.

Xenograft tumor model. A suspension of 500,000 Ishikawa WT or FPS cells in a total volume of 0.2 mL DMEM was injected s.c. into each dorsal flank of CD1-Foxn1tm mice (Charles River). The mice ($n = 30$) were divided into two groups of equal tumor size after engraftment (1 wk). The mice were injected twice weekly with 100 μg IgG (WT and FPS) or CXCL1 neutralizing antibody (FPS) via i.p. injection for 4 wk. One tumor from each mouse was placed in PBS for flow cytometry analysis and RNA extracted from the second tumor from each mouse. The animals were maintained under sterile conditions in individually vented cages.

Flow cytometry analysis. Xenografts from nude mice were assessed for immune cell infiltrate using flow cytometry ($n = 15$). Briefly, tumors were digested by collagenase treatment at 37°C for 45 min. Tissue was then

mechanically disrupted into a single-cell solution using a syringe and 40 μ m mesh and resuspended in fluorescence-activated cell sorting (FACS) wash (PBS + 1% BSA + 2% formalin). Cells were incubated at 4°C for 30 min in FACS wash containing the following monoclonal antibodies and appropriate isotype controls: FITC-CD11b, PE-Gr-1, and Cy5-CD11c. RBC were lysed using BD FACS lysis solution according to manufacturer's instructions (BD Biosciences). Samples were analyzed using a FACScalibur cytometer (BD Biosystems) using BD CellQuest software. Neutrophils were defined by expression of Gr-1 and CD11b epitope, absence of CD11c, and scatter profile.

Statistical analysis. Where appropriate, data were subjected to statistical analysis with ANOVA and Student's *t* test (GraphPad Prism).

Results

CXCL1 expression in FPS cells. Changes in cytokine expression in FPS cells in response to PGF_{2 α} treatment were examined by cytokine antibody array (Fig. 1A). A combined up-regulation of CXCL1, CXCL2, and CXCL3 as well as CXCL1 alone was observed following 100 nmol/L PGF_{2 α} treatment of FPS cells for 24 hours compared with vehicle-treated cells. To verify this finding, the promoter activity (Fig. 1B), mRNA (Fig. 1C), and protein (Fig. 1D) expression of CXCL1 in response to PGF_{2 α} treatment was examined. All were significantly increased ($P < 0.01$) in response to PGF_{2 α} treatment in a time-dependent manner compared with vehicle-treated cells.

Involvement of epidermal growth factor receptor and MEK signaling in CXCL1 production. To determine signaling pathways mediating CXCL1 production in FPS cells, we treated cells with vehicle, 100 nmol/L PGF_{2 α} alone or with a panel of chemical inhibitors of cell signaling, or inhibitor alone (Fig. 2). Treatment of FPS cells with PGF_{2 α} for 8 and 24 hours induced a 91.5- \pm 8.4-fold

and 22.3- \pm 4.7-fold increase in CXCL1 mRNA and protein expression, respectively, compared with vehicle treated cells (Fig. 2A and B). This increase was abolished by treatment of cells with a selective inhibitor of Gq (YM254890; $P < 0.01$) and significantly inhibited with the FP receptor antagonist AL8810 ($P < 0.05$) and inhibitors of epidermal growth factor receptor (EGFR; AG1478; $P < 0.05$) and MEK (PD98059; $P < 0.01$). Inhibitors of calcineurin (CsA) and protein kinase A (43CMQ) did not significantly affect CXCL1 mRNA and protein production.

We confirmed a role for EGFR and extracellular signal-regulated kinase (ERK) in PGF_{2 α} -mediated CXCL1 production by cotransfected FPS cells with the CXCL1 promoter and either an empty vector (pcDNA3.0) or DN EGFR, DNras, DNMEK, or DN-Nuclear factor of activated T-cells (NFAT; Fig. 2C). Treatment of control-vector-transfected cells with 100 nmol/L PGF_{2 α} showed an elevation of CXCL1 promoter activity of 18.9- \pm 3.6-fold, which was significantly reduced by cotransfection of cells with DN-EGFR ($P < 0.05$), DN-ras ($P < 0.01$), and DN-MEK ($P < 0.01$), but no significant difference was shown when cells were transfected with DN-NFAT.

CXCL1 and CXCR2 expression in endometrial adenocarcinoma and normal endometrium. Because we had ascertained a role for the FP receptor in regulating CXCL1 in an endometrial adenocarcinoma cell line, we next investigated the expression and regulation of CXCL1 in endometrial adenocarcinoma explants by PGF_{2 α} . Quantitative RT-PCR analysis showed an increase in the expression of CXCL1 and its receptor CXCR2 mRNA expression in human endometrial adenocarcinoma tissue ($n = 58$) compared with normal endometrium ($n = 45$; 5.9- and 4.2-fold, respectively; $P < 0.001$; Fig. 3A and B). We investigated whether CXCL1 expression in endometrial adenocarcinoma explants was regulated via FP and MEK signaling pathways. Carcinoma tissue was treated

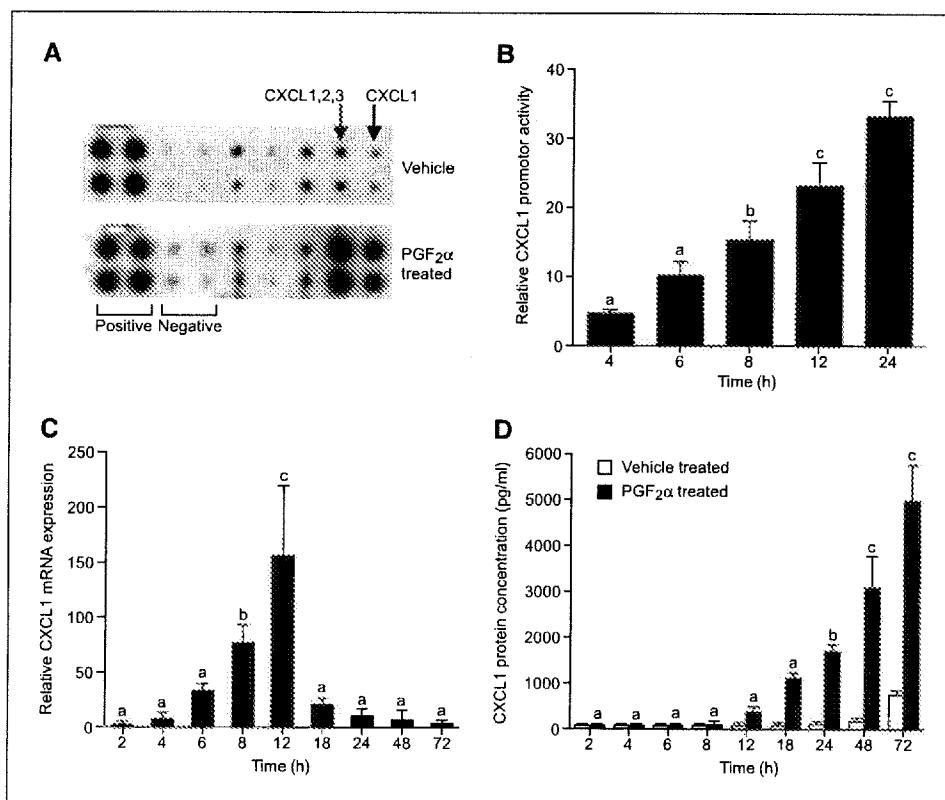


Figure 1. PGF_{2 α} regulates CXCL1 expression in FPS cells. *A*, a human cytokine antibody array was used to determine differences in protein production in the conditioned media of vehicle-treated and PGF_{2 α} -treated cells. *B*, CXCL1 promoter activity was increased in FPS cells stimulated with 100 nmol/L PGF_{2 α} compared with vehicle control over a period of 4 to 24 h. Data are expressed as fold increase in luciferase activity compared with vehicle-treated cells. *C*, FPS cells stimulated with 100 nmol/L PGF_{2 α} show a time-dependent increase in CXCL1 mRNA expression as measured by quantitative RT-PCR. Data are expressed as fold over vehicle-treated cells. *D*, CXCL1 protein secreted into media was measured by ELISA and was increased after treatment with PGF_{2 α} up to 72 h. Columns, mean of at least three independent experiments. $P < 0.05$, *b* is significantly different from *a*; $P < 0.01$, *c* is significantly different from *a* and *b*.

with PGF_{2 α} in the absence/presence of AL8810 and PD98059 for 24 hours. CXCL1 mRNA was found to be elevated 5.3- \pm 0.8-fold in response to PGF_{2 α} ($P < 0.05$). Cotreatment of tissue with AL8810 or PD98059 significantly reduced this increase in CXCL1 expression ($P < 0.05$).

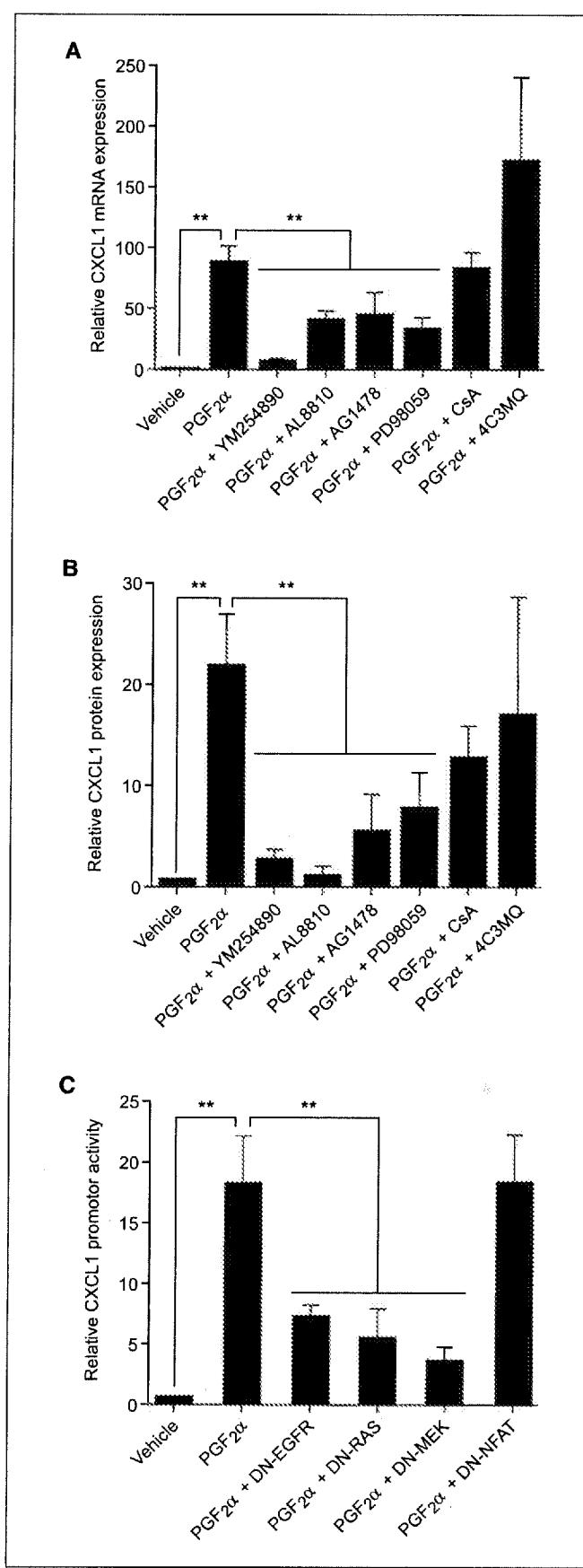
Localization of CXCL1 and CXCR2 in endometrial adenocarcinoma. The site of expression of CXCL1 and CXCR2 protein in carcinoma tissue was then determined by immunohistochemistry (Fig. 4A). CXCL1 and CXCR2 immunoreactivity was localized to glandular epithelium, vascular endothelial cells, and stroma in all well, moderately, and poorly differentiated carcinoma sections studied ($n = 4$ each group).

Using serial sectioning, CXCL1 expression could be localized to the same glandular epithelial and vascular endothelial cells as the FP receptor (Fig. 4B, arrowheads). CXCL1 has been previously described as a potent neutrophil chemoattractant. We next colocalized CXCR2 expression throughout the stroma with expression of neutrophil elastase, a neutrophil marker, in endometrial adenocarcinoma using dual immunofluorescence immunohistochemistry (Fig. 4C). No immunoreactivity was observed in sections incubated with nonimmune IgG. The number of neutrophils present in endometrial tissue was then quantified using immunohistochemistry for neutrophil elastase (Fig. 4D) and was found to be 13.9- \pm 2.3-fold higher in cancer compared with sections of normal endometrium ($P < 0.01$).

PGF_{2 α} -stimulated CXCL1 induces neutrophil chemotaxis *in vitro* and *in vivo*. We next determined whether the CXCL1 expressed in FPS cells via PGF_{2 α} -FP receptor interaction could induce neutrophil chemotaxis. Human neutrophils were purified from peripheral blood and used in a chemotaxis assay. We found a significant increase in neutrophil chemotaxis in response to conditioned media from FPS cells treated with 100 nmol/L PGF_{2 α} (P-CM) compared with vehicle-treated cells (V-CM; Fig. 5A). This effect was significantly inhibited with immunoneutralization of CXCL1 before incubation with neutrophils or with the addition of the CXCR2 antagonist SB225002 to P-CM ($P < 0.001$).

To explore whether FP receptor signaling could promote neutrophil migration *in vivo*, we injected WT or FPS cells s.c. in nude mice. Mice were then regularly injected with control IgG (WT and FPS xenografts) or CXCL1 antibody (FPS xenografts). Tumors formed from FPS cells expressed significantly higher CXCL1 mRNA compared with WT tumors (Fig. 5B) and, when analyzed by flow cytometry, had increased neutrophil infiltration ($P < 0.001$; Fig. 5C). This infiltration was significantly decreased in FPS xenografts injected with CXCL1 neutralizing antibody compared with those treated with nonimmune IgG ($P < 0.001$). This analysis was confirmed further by immunohistochemistry (Fig. 5D), where increased neutrophils were seen distributed throughout FPS xenografts compared with WT or CXCL1 immunoneutralized FPS xenografts.

Figure 2. CXCL1 production in FPS cells is mediated by EGFR, Ras, and MEK. *A*, CXCL1 mRNA production, measured by quantitative RT-PCR, was increased after 8 h of PGF_{2 α} treatment. This was decreased by cotreatment with YM254890, AL8810, AG1478, and PD98059. No reduction was seen after cotreatment with CsA or 4C3MQ. *B*, CXCL1 protein secretion after 24 h of PGF_{2 α} treatment was reduced after cotreatment with YM254890, AL8810, AG1478, and PD98059. No reduction was seen after cotreatment with CsA or 4C3MQ. *C*, CXCL1 promoter activity was measured in cells treated with PGF_{2 α} alone or with DN-EGFR, DN-Ras, DN-MEK, or DN-NFAT. Significant reduction in activity was seen after 8 h with all constructs except DN-NFAT. Columns, mean of at least three independent experiments; **, $P < 0.01$.



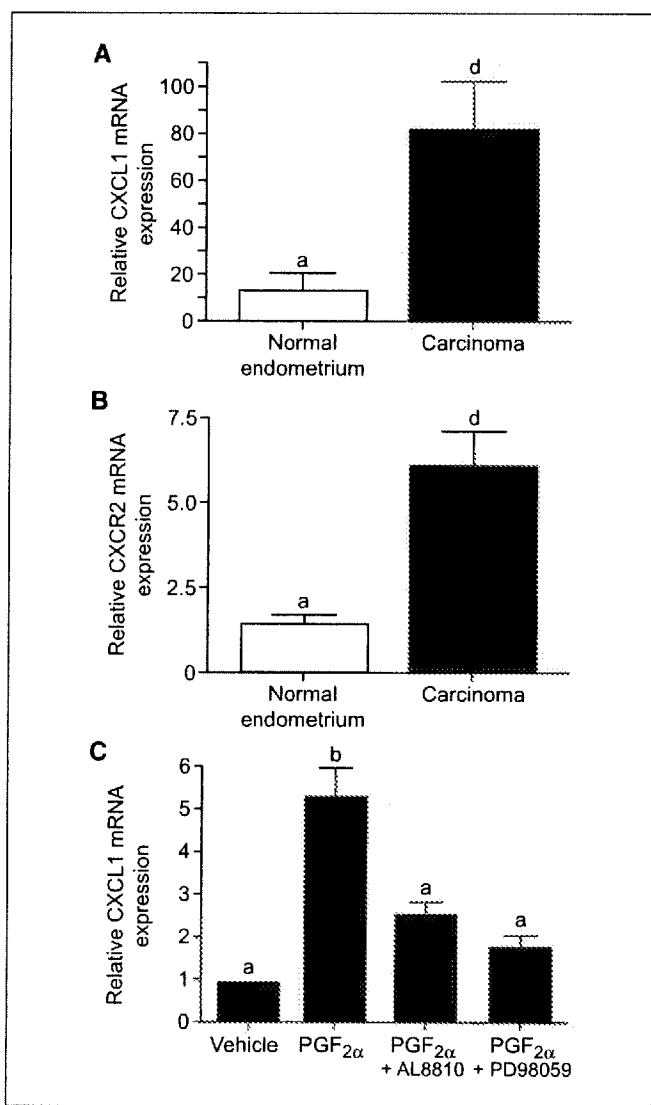


Figure 3. CXCL1 and CXCR2 expression is increased in endometrial adenocarcinoma. CXCL1 (A) and CXCR2 (B) mRNA expression is significantly increased in endometrial adenocarcinoma tissue ($n = 58$) compared with normal endometrium ($n = 45$). C, CXCL1 mRNA is increased in endometrial adenocarcinoma explants ($n = 4$) after treatment with PGF_{2α}. Cotreatment with AL8810 or PD98059 significantly decreases this effect. $P < 0.05$, *b* is significantly different from *a*; $P < 0.001$, *d* is significantly different from *a* and *b*.

Discussion

The link between inflammation and tumor progression has been shown in a range of studies. For example, elevated expression of inflammatory COX-2 and prostaglandins has been correlated with tumor growth and angiogenesis in prostate, pancreatic, and colon cancer (31–33), and the risk of long-term inflammation has been shown by studies showing that continued use of specific COX-2 inhibitors nonsteroidal anti-inflammatory drugs can significantly reduce cancer occurrence in patients at high risk (34). In the present study, we show that PGF_{2α}-FP signaling can regulate expression of the inflammatory chemokine CXCL1 in endometrial adenocarcinoma cells to modulate neutrophil influx in tumors. To our knowledge, this is the first study to provide a link between inflammatory prostanoids,

specifically PGF_{2α}, and neutrophil recruitment in endometrial cancers.

Prostaglandins have been shown to regulate chemokine expression *in vitro* (35, 36). Prostaglandin E₂ is overexpressed in many cancer types and has been shown to induce CXCL1 production in colon cancer cells, which can then promote tube formation and migration of endothelial cells (14). We have previously ascertained a role for the FP receptor and PGF_{2α} signaling in regulating endometrial adenocarcinoma (8–11). In the present study, we investigated a role for the FP receptor in modulating the expression of chemokines using an *in vitro* model system of Ishikawa cells stably expressing the human FP receptor (FPS cells; ref. 9) and a human cytokine antibody array. The array identified CXCL1 as a key cytokine induced by PGF_{2α}-FP signaling. Using FPS cells, which we have previously shown to reproduce the *ex vivo* effects of PGF_{2α} on endometrial adenocarcinoma tissue explants (9), we elaborated the signaling pathways mediating the role of FP on CXCL1 expression using chemical inhibitors and DN mutants of cell signaling pathways. A key effector pathway that has been previously shown to regulate tumorigenic signaling molecules in response to G-protein-coupled receptor signaling is the mitogen-activated protein kinase (MAPK) pathway. The signaling components of this pathway in FPS cells have been identified in our laboratory, where the phosphorylation of the downstream component of the MAPK pathway, ERK1/2, was shown to be mediated by EGFR *trans*-activation and c-Src phosphorylation (9). We found that chemical inhibitors of EGFR and MEK could inhibit CXCL1 production, as did cotransfection of DN EGFR, Ras, and MEK. However, NFAT, a common regulator of cytokine expression (37), was not involved in PGF_{2α}-mediated CXCL1 production in this cell type. These data are supported by previously published evidence in colorectal adenocarcinoma cell lines where the ERK pathway was also shown to be crucial in the regulation of CXCL1 expression after stimulation with prostaglandin E₂ (14).

Overexpression of CXCL1 has previously been shown in a variety of tumor types, including colorectal (18) and melanoma (15), and promotes a variety of cellular functions including cell proliferation in esophageal cancer (38) and cell invasion in bladder cancer (39). Here, we showed elevated expression of CXCL1 and its receptor CXCR2 in endometrial adenocarcinoma compared with normal endometrium. Expression of both was localized to glandular epithelium, stroma, and vascular endothelial cells. In addition, treatment of endometrial adenocarcinoma explants with PGF_{2α} caused an increase in CXCL1 expression via FP receptor and ERK1/2 signaling pathways confirming the importance of this signaling cascade in regulating CXCL1 expression *ex vivo*. CXCR2 localization in neutrophils in endometrial adenocarcinoma suggested that CXCL1 via CXCR2 could play a role in immune cell function. A role for CXCL1 in neutrophil influx has been previously shown in an angiogenic sponge model in the mouse, as endogenous CXCL1 expression increased immediately preceding a neutrophil influx (40).

Here, we show by immunohistochemistry that neutrophils are elevated in human endometrial adenocarcinomas. We have also confirmed that CXCL1 is strongly chemotactic to neutrophils as conditioned media from PGF_{2α}-stimulated FPS cells induced chemotaxis of peripheral neutrophils. This chemotaxis was significantly reduced by CXCL1 immunoneutralization and CXCR2 inhibition using a specific antagonist. To determine a role for CXCL1 induced by PGF_{2α}-FP interaction *in vivo*, we inoculated nude mice with FPS and WT cells. The increased neutrophils in the resulting FPS tumors compared with WT were significantly reduced

by injection of CXCL1 neutralizing antibodies, demonstrating that PGF_{2 α} signaling via CXCL1 is influencing neutrophil cell infiltration in endometrial adenocarcinomas. Neutrophil infiltration into tumors has also been shown to be dependent on CXC chemokine-CXCR2 signaling in a model of melanoma in a CXCR2 null nude mouse (41).

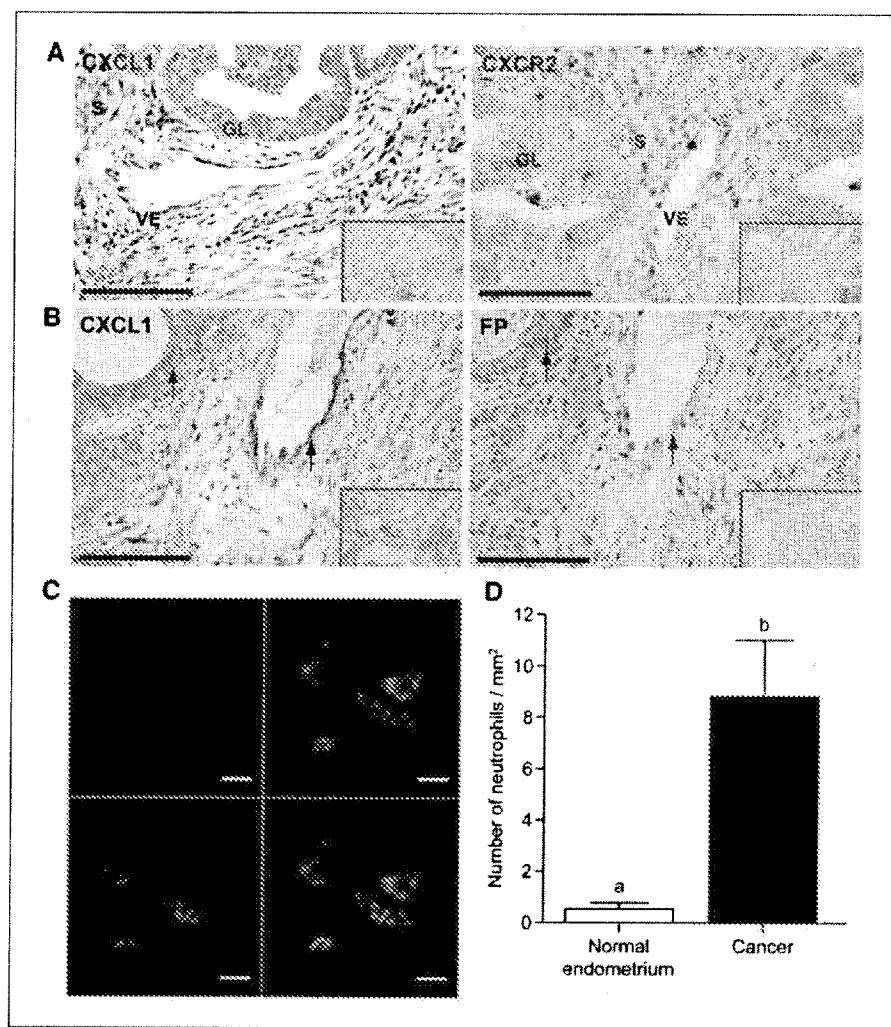
A chemokine-mediated influx of neutrophils is seen in the late secretory phase of the normal endometrium (42). Their role may be dependent on the activating agents and cytokines present, but they are thought to be involved in the breakdown and repair at menstruation by degranulation and the release of proteases that degrade the extracellular matrix (43). They may also be capable of remodeling vasculature, as neutrophils found close to or associated with endothelial microvessels express VEGF during or coincident with angiogenesis in the normal menstrual cycle (44).

The role of neutrophils in endometrial adenocarcinoma is unclear, and in our study, similar to other reports (45, 46), neutrophil influx in our xenograft model did not effect on tumor size. However, considering their profound tissue-remodeling capabilities, which have been shown in a number of animal models of other cancer types, it is possible that they play a similar role in endometrial cancer. For example, neutrophils have been shown to uniquely produce a tissue inhibitor of metalloproteinase-free MMP-9, a key protease involved in extracellular matrix

degradation, which may affect the tumor microenvironment by tissue remodeling (24). In addition, the depletion of neutrophils in a mouse model was shown to prevent metastasis of fibrosarcoma cells from the primary tumor (45), suggesting a role for neutrophils in the switch to a metastatic phenotype. In a nude mouse model of breast cancer, overexpression of interleukin-8, a chemokine related to CXCL1, caused an infiltration of neutrophils, which increased invasiveness of the tumor, likely due to an increase in protease production (47). Similarly, the decrease in neutrophil infiltration caused by an inhibition of CXCL1 expression in a nude mouse model of colon cancer significantly decreased metastasis in these animals (48). Furthermore, neutrophils may also promote tumorigenesis through means other than tissue remodeling. In an *in vitro* model of colon cancer, neutrophils promote cellular stress by inducing transient errors in DNA replication in epithelial cells (49), which could ultimately lead to carcinogenesis, whereas neutrophils from ovarian cancer patients released higher levels of reactive oxygen species, which could potentially lead to cellular changes that support tumor progression (50).

In conclusion, we provide evidence for a novel PGF_{2 α} -FP pathway that can regulate the inflammatory microenvironment in endometrial adenocarcinoma via CXCL1-induced neutrophil chemotaxis.

Figure 4. CXCL1 and CXCR2 are expressed in endometrial adenocarcinoma and colocalized with FP receptor expression and neutrophil elastase, respectively. *A*, immunohistochemical staining of CXCL1 and CXCR2 in moderately and poorly differentiated endometrial adenocarcinoma. *GL*, glandular; *VE*, vascular endothelial; *S*, stromal. *B*, CXCL1 can be colocalized to the same cells as FP receptor (arrowheads) in well-differentiated adenocarcinoma by serial sectioning. *C*, colocalization of the site of expression of CXCR2 (green) and neutrophil elastase (red). *Inset*, negative controls are inset. *D*, carcinoma sections ($n = 30$) and normal proliferative endometrium ($n = 7$) were analyzed for expression of neutrophil elastase. Increased expression was seen in cancer (*b* is significantly different from *a*; $P < 0.01$). Scale bars, 100 μ m (*A* and *B*) and 10 μ m (*C*).



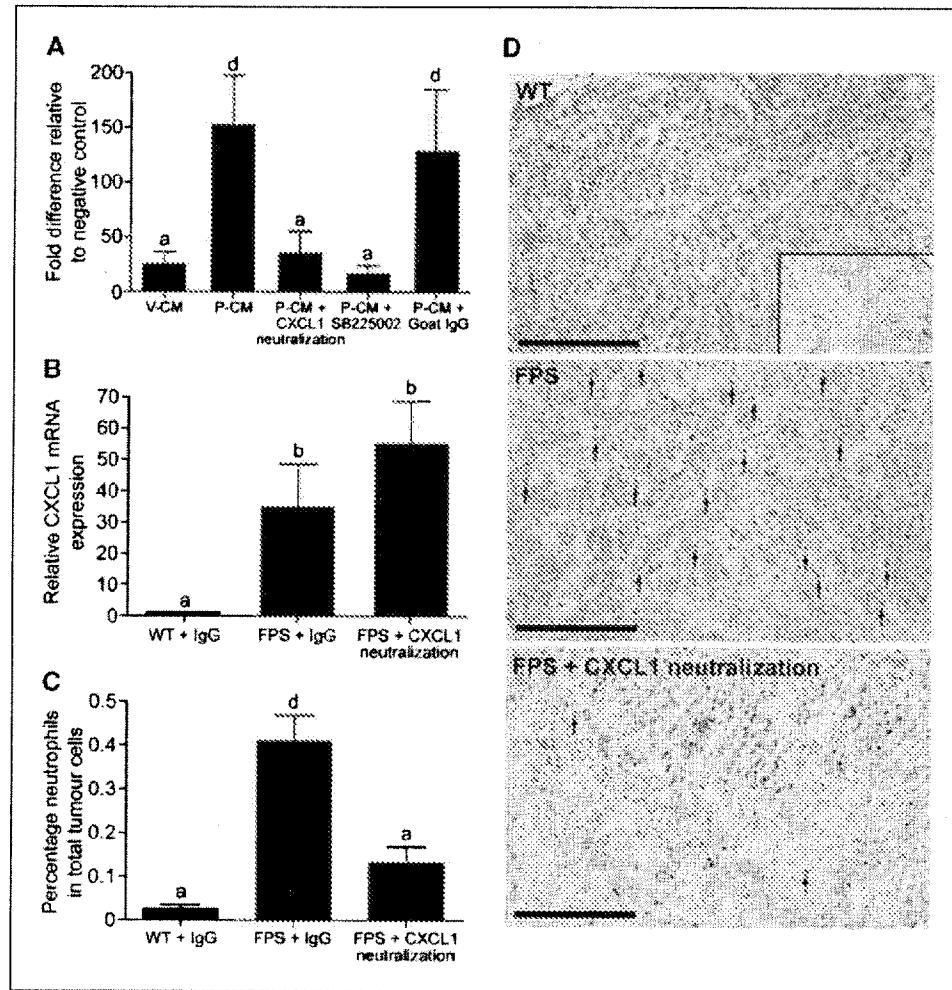


Figure 5. Neutrophils migrate in response to PGF_{2α}-induced CXCL1 *in vitro* and *in vivo*. *A*, neutrophil chemotaxis was increased in response to conditioned media from FPS cells treated with PGF_{2α} for 48 h (P-CM) compared with vehicle-treated cells (V-CM). CXCL1 immunoneutralization and the addition of 60 nmol/L SB-225002 significantly decreased chemotaxis. Control goat IgG immunoneutralization showed no significant effect. Data are expressed as fold over negative control (serum-free medium). *B*, CXCL1 mRNA expression is significantly increased in nude mice xenografts formed from FPS cells compared with WT cell xenografts. Injection of CXCL1 antibody into xenografts made no significant difference to CXCL1 mRNA expression. *C*, percentage of neutrophils in tumors from WT and FPS xenografts ($n = 5$ per group), measured by flow cytometry. Increased neutrophil infiltration is seen in FPS xenografts, which is significantly reduced by injection of CXCL1-neutralizing antibody. *D*, localization of neutrophils in xenografts by Gr-1 staining. Arrowheads, neutrophils; scale bars, 100 μ m ($P < 0.05$, *b* is significantly different from *a*; $P < 0.01$, *c* is significantly different from *a* and *b*; $P < 0.001$, *d* is significantly different from *a*, *b*, and *c*).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 2/2/09; revised 5/1/09; accepted 5/8/09; published OnlineFirst 6/23/09.

Grant support: Medical Research Council core funding (H.N. Jabbour; U.1276.00.0004.00002.01).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Anne Saunderson for patient recruitment and tissue collection, and Professor S. Howie, Dr. S. Battersby, S. Wright, V. Grant, and M. Keightley for helpful discussion and technical assistance.

References

- Doll A, Abal M, Rigau M, et al. Novel molecular profiles of endometrial cancer-new light through old windows. *J Steroid Biochem Mol Biol* 2008;108:221–9.
- Jabbour HN, Sales KJ, Smith OP, Battersby S, Boddy SC. Prostaglandin receptors are mediators of vascular function in endometrial pathologies. *Mol Cell Endocrinol* 2006;252:191–200.
- Cha YI, DuBois RN. NSAIDs and cancer prevention: targets downstream of COX-2. *Annu Rev Med* 2007;58:239–52.
- Hofer G, Bieglmayer C, Kopp B, Janisch H. Measurement of eicosanoids in menstrual fluid by the combined use of high pressure chromatography and radioimmunoassay. *Prostaglandins* 1993;45:413–26.
- Reid G, Wielinga P, Zelcer N, et al. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proc Natl Acad Sci U S A* 2003;100:9244–9.
- Abramovitz M, Boie Y, Nguyen T, et al. Cloning and expression of a cDNA for the human prostanoid FP receptor. *J Biol Chem* 1994;269:2632–6.
- Jabbour HN, Sales KJ, Boddy SC, Anderson RA, Williams AR. A positive feedback loop that regulates cyclooxygenase-2 expression and prostaglandin F_{2α} synthesis via the F-series-prostanoid receptor and extracellular signal-regulated kinase 1/2 signaling pathway. *Endocrinology* 2005;146:4657–64.
- Sales KJ, Boddy SC, Williams AR, Anderson RA, Jabbour HN. F-prostanoid receptor regulation of fibroblast growth factor 2 signaling in endometrial adenocarcinoma cells. *Endocrinology* 2007;148:3635–44.
- Sales KJ, List T, Boddy SC, et al. A novel angiogenic role for prostaglandin F_{2α}-FP receptor interaction in human endometrial adenocarcinomas. *Cancer Res* 2005;65:7707–16.
- Sales KJ, Milne SA, Williams AR, Anderson RA, Jabbour HN. Expression, localization, and signaling of prostaglandin F_{2α} receptor in human endometrial adenocarcinoma: regulation of proliferation by activation of the epidermal growth factor receptor and mitogen-activated protein kinase signaling pathways. *J Clin Endocrinol Metab* 2004;89:986–93.
- Sales KJ, Boddy SC, Jabbour HN. F-prostanoid receptor alters adhesion, morphology and migration of endometrial adenocarcinoma cells. *Oncogene* 2008;27:2466–77.
- Milne SA, Jabbour HN. Prostaglandin (PG) F_(2α) receptor expression and signaling in human endometrium: role of PGF(2α) in epithelial cell proliferation. *J Clin Endocrinol Metab* 2003;88:1825–32.
- Strieter RM, Burdick MD, Mestas J, Gomperts B, Keane MP, Belperio JA. Cancer CXC chemokine networks and tumor angiogenesis. *Eur J Cancer* 2006;42:768–78.

14. Wang D, Wang H, Brown J, et al. CXCL1 induced by prostaglandin E2 promotes angiogenesis in colorectal cancer. *J Exp Med* 2006;203:941–51.

15. Haghnegahdar H, Du J, Wang D, et al. The tumorigenic and angiogenic effects of MGSA/GRO proteins in melanoma. *J Leukoc Biol* 2000;67:53–62.

16. Wang D, Yang W, Du J, et al. MGSA/GRO-mediated melanocyte transformation involves induction of Ras expression. *Oncogene* 2000;19:4647–59.

17. Li A, Varney ML, Singh RK. Constitutive expression of growth regulated oncogene (GRO) in human colon carcinoma cells with different metastatic potential and its role in regulating their metastatic phenotype. *Clin Exp Metastasis* 2004;21:571–9.

18. Wen Y, Giardina SF, Hamming D, et al. GROα is highly expressed in adenocarcinoma of the colon and down-regulates fibulin-1. *Clin Cancer Res* 2006;12:5951–9.

19. Moore BB, Arentzen DA, Stoy K, et al. Distinct CXC chemokines mediate tumorigenicity of prostate cancer cells. *Am J Pathol* 1999;154:1503–12.

20. Addison CL, Daniel TO, Burdick MD, et al. The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR⁺ CXC chemokine-induced angiogenic activity. *J Immunol* 2000;165:5269–77.

21. Moser B, Clark-Lewis I, Zwahlen R, Baggolini M. Neutrophil-activating properties of the melanoma growth-stimulatory activity. *J Exp Med* 1990;171:1797–802.

22. Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 2000;103:481–90.

23. Scapini P, Morini M, Tecchio C, et al. CXCL1/macrophage inflammatory protein-2-induced angiogenesis *in vivo* is mediated by neutrophil-derived vascular endothelial growth factor-A. *J Immunol* 2004;172:5034–40.

24. Ardi VC, Kupriyanova TA, Deryugina EI, Quigley JP. Human neutrophils uniquely release TIMP-free MMP-9 to provide a potent catalytic stimulator of angiogenesis. *Proc Natl Acad Sci U S A* 2007;104:20262–7.

25. Sales KJ, Grant V, Jabbour HN. Prostaglandin E2 and F2α activate the FP receptor and up-regulate cyclooxygenase-2 expression via the cyclic AMP response element. *Mol Cell Endocrinol* 2008;285:51–61.

26. Nirodi C, NagDas S, Gygi SP, Olson G, Aebersold R, Richmond A. A role for poly(ADP-ribose) polymerase in the transcriptional regulation of the melanoma growth stimulatory activity (CXCL1) gene expression. *J Biol Chem* 2001;276:9366–74.

27. Levi NL, Hanoch T, Benard O, et al. Stimulation of Jun N-terminal kinase (JNK) by gonadotropin-releasing hormone in pituitary α T3-1 cell line is mediated by protein kinase C, c-Src, and CDC42. *Mol Endocrinol* 1998;12:815–24.

28. Harris D, Bonfil D, Chuderland D, Kraus S, Seger R, Naor Z. Activation of MAPK cascades by GnRH: ERK and Jun N-terminal kinase are involved in basal and GnRH-stimulated activity of the glycoprotein hormone LHβ-subunit promoter. *Endocrinology* 2002;143:1018–25.

29. Lan HY, Mu W, Nikolic-Paterson DJ, Atkins RC. A novel, simple, reliable, and sensitive method for multiple immunoenzyme staining: use of microwave oven heating to block antibody crossreactivity and retrieve antigens. *J Histochem Cytochem* 1995;43:97–102.

30. Ward C, Chilvers ER, Lawson MF, et al. NF-κB activation is a critical regulator of human granulocyte apoptosis *in vitro*. *J Biol Chem* 1999;274:4309–18.

31. Molina MA, Sitja-Arnau M, Lemoine MG, Frazier ML, Sinicrope FA. Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by nonsteroidal anti-inflammatory drugs. *Cancer Res* 1999;59:4356–62.

32. Tsuji M, Kawano S, Tsuji S, Saewaka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* 1998;93:705–16.

33. Jain S, Chakraborty G, Raju R, Kale S, Kundu GC. Prostaglandin E2 regulates tumor angiogenesis in prostate cancer. *Cancer Res* 2008;68:7750–9.

34. Dannenberg AJ, Subbaraoaiah K. Targeting cyclooxygenase-2 in human neoplasia: rationale and promise. *Cancer Cell* 2003;4:431–6.

35. Pold M, Zhu LX, Sharma S, et al. Cyclooxygenase-2-dependent expression of angiogenic CXC chemokines ENA-78/CXC Ligand (CXCL) 5 and interleukin-8/CXCL8 in human non-small cell lung cancer. *Cancer Res* 2004;64:1853–60.

36. Takehara H, Iwamoto J, Mizokami Y, et al. Involvement of cyclooxygenase-2-prostaglandin E2 pathway in interleukin-8 production in gastric cancer cells. *Dig Dis Sci* 2006;51:2188–97.

37. Boyd JH, Divangahi M, Yahiaoui L, Gvozdic D, Qureshi S, Petrof BJ. Toll-like receptors differentially regulate CC and CXC chemokines in skeletal muscle via NF-κB and calcineurin. *Infect Immun* 2006;74:6829–38.

38. Wang B, Hendricks DT, Wamunyokoli E, Parker MI. A growth-related oncogene/CXC chemokine receptor 2 autocrine loop contributes to cellular proliferation in esophageal cancer. *Cancer Res* 2006;66:3071–7.

39. Kawanishi H, Matsui Y, Ito M, et al. Secreted CXCL1 is a potential mediator and marker of the tumor invasion of bladder cancer. *Clin Cancer Res* 2008;14:2579–87.

40. Barcelos LS, Talvani A, Teixeira AS, Cassali GD, Andrade SP, Teixeira MM. Production and *in vivo* effects of chemokines CXCL1–3/KC and CCL2/JE in a model of inflammatory angiogenesis in mice. *Inflamm Res* 2004;53:576–84.

41. Singh S, Varney M, Singh RK. Host CXCR2-dependent regulation of melanoma growth, angiogenesis, and experimental lung metastasis. *Cancer Res* 2009;69:411–5.

42. Critchley HO, Kelly RW, Kooy J. Perivascular location of a chemokine interleukin-8 in human endometrium: a preliminary report. *Hum Reprod* 1994;9:1406–9.

43. Kaitu'u-Lino TJ, Morison NB, Salamonsen LA. Neutrophil depletion retards endometrial repair in a mouse model. *Cell Tissue Res* 2007;328:197–206.

44. Gargett CE, Lederman E, Heryanto B, Gambino LS, Rogers PA. Focal vascular endothelial growth factor correlates with angiogenesis in human endometrium. Role of intravascular neutrophils. *Hum Reprod* 2001;16:1065–75.

45. Tazawa H, Okada F, Kobayashi T, et al. Infiltration of neutrophils is required for acquisition of metastatic phenotype of benign murine fibrosarcoma cells: implication of inflammation-associated carcinogenesis and tumor progression. *Am J Pathol* 2003;163:2221–32.

46. Shojaei F, Singh M, Thompson JD, Ferrara N. Role of Bv8 in neutrophil-dependent angiogenesis in a transgenic model of cancer progression. *Proc Natl Acad Sci U S A* 2008;105:2640–5.

47. Yao C, Lin Y, Chua MS, et al. Interleukin-8 modulates growth and invasiveness of estrogen receptor-negative breast cancer cells. *Int J Cancer* 2007;121:1949–57.

48. Yamamoto M, Kikuchi H, Ohta M, et al. TSU68 prevents liver metastasis of colon cancer xenografts by modulating the premetastatic niche. *Cancer Res* 2008;68:9754–62.

49. Campregher C, Luciani MG, Gasche C. Activated neutrophils induce an hMSH2-dependent G2/M checkpoint arrest and replication errors at a (CA)13-repeat in colon epithelial cells. *Gut* 2008;57:780–7.

50. Klink M, Jastrzembska K, Nowak M, et al. Ovarian cancer cells modulate human blood neutrophils response to activation *in vitro*. *Scand J Immunol* 2008;68:328–36.